REMARKS

Upon entry of the present amendment, claims 22 and 23 will be pending. Claims 22 and 23 have been amended to clarify their scope. Support for the amendments can be found in the specification, for example, at ¶[0070] and ¶[0072] and in the Examples. No new matter has been added.

Interview Summary

Applicants and their representatives sincerely thank Examiner Noble for her time, courtesy, and constructive comments during the interview of December 31, 2009 with the undersigned. During the interview, the Applicants' undersigned representative and the Examiner discussed and clarified the enablement rejection. In accord with the Examiner's suggestion, the arguments made during the interview have been incorporated into the remarks below.

Applicants respectfully request entry of the above amendment and allowance of the claims in view of the remarks in this Response.

35 U.S.C. §112, first paragraph

The Examiner has rejected claims 22 and 23 for alleged lack of enablement. As the Applicants understand it, the Examiner bases her rejection on three grounds:

(1) that claims 22 and 23 encompassed the use of the method for producing antibodies in "any virus" (Office Action at page 5); and

(2) her concern that one of skill in the art would have to undertake undue experimentation in order to use an immunogen comprising "a fraction of a virus" (Office Action at page 3);

(3) her concern that one of skill in the art would have to undertake undue experimentation in order to use an immunogen comprising "a baculovirus that does not express PepT1 or the antigen on the surface of the virus" (Office Action at page 3).

Applicants believe that the above amendment of claims 22 and 23 to specify that the budding virus is a baculovirus fully addresses and overcomes part (1) of the enablement rejection.

Applicants respectfully traverse parts (2) and (3) of the enablement rejection. An invention, as defined by the claims, is enabled where one of ordinary skill in the art can make and use it without resorting to undue experimentation. See, *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed.Cir.1988). All of the evidence must be considered, and any conclusion of nonenablement must be based on the evidence as a whole. MPEP § 2164.01(a).

Contrary to the Examiner's assertion, one of skill in the art would be able to practice the claimed methods of producing an antibody without undue experimentation. The Examiner appears to require that any method of making antibodies in the gp64 transgenic mice of the claims be necessarily limited to a target antigen, *e.g.*, PepT1, that is present both on an intact budding baculovirus particle and that is displayed on the surface of the baculovirus particle that is administered to the gp64 transgenic animals. The Examiner states that:

> The specification fails to teach how to introduce the target antigen in context of a fraction of a baculovirus. The specification fails to teach how to immunize without the target antigen being expressed by the baculovirus. The specification fails to teach how to introduce the target antigen expressed inside the baculoviral particle and not with the envelope proteins on the surface of the particle. (Office Action at page 5.)

As the Examiner recognizes, the method is premised on the use of immunogens that are expressed in a baculovirus. Applicants remind the Examiner that the general problem the inventors set out to solve was to develop a method of efficiently producing antibodies against virally expressed target antigens by reducing or eliminating the production of antibodies to highly immunogenic background viral antigens that typically contaminate virally-expressed target antigens. To that end, the inventors set out to develop, and did develop, mice that were transgenic for the soluble form of one such background antigen, gp64. As the Applicants have disclosed, the gp64 transgenic mice showed immunologic tolerance to gp64 (See Example 8). Thus, the methods of the amended claims allow one of skill in the art to efficiently generate specific antibodies to a desired protein using an immunogen that contains contaminating background antigens, *e.g.*, gp64.

The specification provides substantial guidance regarding the immunogen. The specification describes immunogens in many forms, *e.g.*, baculoviral particles and fractions of

baculoviral particles, and makes clear that the common feature shared by all these forms is not display on the surface of an intact baculovirus, but rather *the presence of contaminating background antigens*:

> Immunogens of the present invention specifically include cells, cell cultures, cell lysates, viruses, or unpurified antigens. *Parts of cells or viruses can be used as immunogens, as well as whole cells or whole viruses. For example, cell membranes or virus envelopes can be used as immunogens.* When a cell or virus is used as an immunogen, a gene coding for a subject antigen can be artificially introduced into the cell or virus by recombinant gene technology that artificially expresses the subject antigen.
>
> One preferable immunogen of the present invention is *a viral particle or part thereof.* Viruses are comprised of relatively simple components, including nucleic acids, and limited proteins, saccharides, and such. Consequently, the types of background antigens that may interfere with target antigen isolation are also limited. In sum, inducing immunotolerance against a limited number of background antigens in an animal to be immunized would be enough to carry out a method for producing antigen of the present invention. (Specification at page 10, lines 32-36 and page 11, lines 1-11, emphasis added.)

Upon reading the Applicants' specification, one of skill in the art would recognize that the method of the claims could be used for *any* immunogen that was contaminated with background gp64 protein. What is claimed is a method for the efficient production of antibodies to a specific immunogen when that immunogen happens to be contaminated with highly immunogenic gp64 protein. Immunization with partially purified proteins or cell lysates is a method that is routine in the art and one of ordinary skill would readily understand that while the method of the claims could be used for immunogens that included intact baculoviral particles that expressed the target antigen on their surface, it is equally applicable to immunogens comprising a fraction of a baculoviral particle, *e.g.*, lysates, extracts or partially purified fractions of those same particles as long as they contained the target antigen. Moreover, while successful immunization with intact baculovirus particles requires the surface display of the target antigen, immunization with baculoviral fractions is not nearly so restricted and can result in the production of specific antibodies regardless of the localization of the corresponding target antigens in the intact particle. In fact, surface localization of the target antigens is largely

irrelevant when the immunogen is a baculoviral lysate. In an intact particle, only those proteins on the surface have access to the immune system; once the particle has been fractionated, *all* proteins, including those found on the inside of the particle become potential targets for recognition by the immune system.

The art recognized at the time the application was filed that baculoviral particles that had been engineered to include one or more recombinant proteins could serve as a useful source of starting material for purification of those proteins. Methods of preparing a fraction of a baculoviral particle are described, for example, in Hayashi *et al.*, *Journal of Biological Chemistry* 279: 38040-38046 (2004) ("Hayashi"), U.S. Patent 6,713,278 (document "AD" in the Information Disclosure Statement submitted on October 24, 2006) (the '278 patent) and U.S. Patent 7,070,978 (the '978 patent) which are attached as Exhibits A, B and C, respectively. Each of these references discloses a different method of preparing a fraction of a baculovirus particle; Hayashi (see page 38041, right hand column, lines 1-5) solubilized particles with 1% CHAPSO in HEPES buffer; the '278 patent discloses solubilization in 0.3% n-dodecyl maltoside in 10 mM Tris-HCL, 100 mM NaCl, 2 mM EDTA, pH 7.4 (see column 6, lines 23-28); and the '978 patent discloses solubilization in lyso-phosphatidylcholine (see column 8, lines 4-8). One of skill in the art would readily appreciate not only that the method of the claims encompassed the use of fractionated baculovirus particles or cell lysates, but would be well aware of methods for preparing such fractions.

Thus, the method of the claims has broader applicability than the Examiner asserts and there is no reason to limit the scope of the claims to that proposed by the Examiner on page 3 of the Office Action (*i.e.*, "(a) immunizing a transgenic mouse with an immunogen comprising a budding virus or fraction thereof, wherein the baculovirus where (sic) expresses PepT1 or a fragment thereof on the surface of the baculovirus" (emphasis added by Examiner)). The claimed method can be used to generate antibodies against any target antigen as long as it is administered in the context of a baculovirus particle or portion thereof that includes gp64.

Applicants submit that the specification enables one of ordinary skill in the art, as of the effective filing date, to make and use the invention of claims 22 and 23 without resort to undue

experimentation. In view of the foregoing, the Office is respectfully asked to reconsider and withdraw this ground for rejection.

Applicants submit that all claims are now in condition for allowance, and such action is requested. Please charge any required fees and apply any other charges or credits to deposit account 06-1050 referencing attorney docket number 14875-0137US1.

Respectfully submitted,

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EXHIBIT A

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Selective Reconstitution and Recovery of Functional γ-Secretase Complex on Budded Baculovirus Particles*[S]

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***In vitro* reconstitution of functions of membrane proteins is often hampered by aggregation, misfolding, or lack of post-translational modifications of the proteins attributable to overexpression. To overcome this technical obstacle, we have developed a method to express multimeric integral membrane proteins in extracellular (budded) baculovirus particles that are released from Sf9 cells co-infected with multiple transmembrane proteins. We applied this method to the reconstitution of γ-secretase, a membrane protease complex that catalyzes the intramembrane cleavage of β-amyloid precursor protein to release Aβ peptides, the major component of amyloid deposits in Alzheimer brains as well as of Notch. When we co-infected Sf9 cells with human presenilin 1 (PS1), nicastrin, APH-1a, and PEN-2, a high-molecular-weight membrane protein complex that contained PS1 exclusively in its fragment form associated with three other cofactor proteins was reconstituted and recovered in a highly γ-secretase-active state in budded virus particles, whereas nonfunctional PS1 holoproteins massively contaminated the parental Sf9 cell membranes. The relative γ-secretase activity (per molar PS1 fragments) was concentrated by ~2.5 fold in budded virus particles compared with that in Sf9 membranes. The budded baculovirus system will facilitate structural and functional analyses of γ-secretase, as well as screening of its binding molecules or inhibitors, and will also provide a versatile methodology for the characterization of a variety of membrane protein complexes.**

A wide variety of protein expression systems using heterologous organisms have been applied to the structural and functional analyses of multispan membrane proteins. However, overexpression and recovery of membrane proteins in a biologically active state are often hampered by the following problems. First, a significant proportion of overexpressed proteins tend to misfold and aggregate without forming proper tertiary structures. Second, lack of appropriate post-translational modifications, interaction with binding-partner proteins, or proper intracellular transport render the membrane proteins stuck within the early compartments of intracellular membrane trafficking. Furthermore, serious contamination of biologically inactive, nascent, or misfolded proteins is inevitable, which makes the analysis of the functional form of protein difficult.

The infection of recombinant baculovirus into *Spodoptera frugiperda* insect cells (Sf9) is one of the most widely used methodologies that achieves a high level of expression and yield of recombinant membrane proteins (1). Baculovirus produces budded virus (BV),[1] *i.e.* particles that contain viral DNA enveloped within Sf9 cell-derived membranes, during its life cycle (Fig. 1). Recently, it has been reported that a seventh membrane-spanning, G protein-coupled receptor was successfully recovered in BV in a functional form complexed with heterotrimeric G proteins and adenylyl cyclase (2, 3). Using this strategy, sterol regulatory element-binding protein-2 (SREBP) and SREBP cleavage-activating protein, which are endoplasmic reticulum-resident membrane proteins in mammalian cells, also were displayed on BV particles with a lesser extent of aggregation and degradation compared with those in cellular membrane fractions (4). However, selective reconstitution of a protein complex composed of multiple integral membrane proteins in a biologically active state has not been achieved yet.

γ-Secretase is a membrane-bound protease complex composed of at least four transmembrane proteins, *i.e.* presenilin (PS), which is predicted to harbor the catalytic center as an aspartic protease, as well as nicastrin (NCT), APH-1, and PEN-2 as essential transmembrane cofactor proteins (5, 6). γ-Secretase is responsible for the intramembrane cleavage of a subset of type 1, single-span membrane proteins (7), including β-amyloid precursor protein (βAPP), to form amyloid β peptides (Aβ); Aβ is the major component of amyloid deposits in the brains of patients with Alzheimer's disease and is closely linked to the pathogenesis of Alzheimer's disease (8). Another important substrate of γ-secretase is Notch, the intracellular domain (ICD) of which is released by γ-secretase and enters the nucleus, thereby transducing intercellular information during tissue development and renewal through transactivation of gene expression (8). Overexpression of the four putative

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[S] The on-line version of this article (available at http://www.jbc.org) contains Fig. 1S.

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[1] The abbreviations used are: BV, budded virus; SREBP, sterol regulatory element-binding protein-2; PS, presenilin; NCT, nicastrin; βAPP, amyloid-β precursor protein; Aβ, amyloid β peptide; ICD, intracellular domain; HMW, high molecular weight; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester; ELISA, enzyme-linked immunosorbent assay.

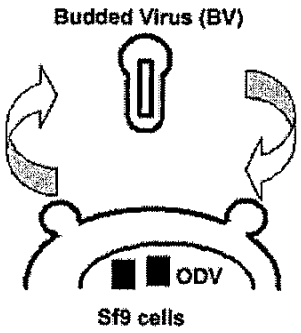

FIG. 1. **Life cycle of baculovirus and generation of BV.** Baculovirus undergoes a biphasic life cycle in its lepidopteron host. Intracellular copies of viral DNA are targeted into BV or alternatively incorporated within occlusion-derived virus (*ODV*) within the nucleus.

γ-secretase components in mammalian or *Drosophila* cells led to the maturation of γ-secretase protein complex, *i.e.* endoproteolysis of PS to generate fragments (the amino-terminal fragment and the carboxyl-terminal fragment, respectively) and high-molecular-weight (HMW) complex formation, as well as an up-regulation of the proteolytic activity (9–12). Importantly, overexpression of all four components in *Saccharomyces cerevisiae*, which lacks endogenous γ-secretase activity, successfully reconstituted the γ-secretase activity (13), strongly suggesting that a membrane protein complex composed of these four proteins represents the minimal framework of γ-secretase.

Selective recovery and purification of the active form of γ-secretase complex is essential to its functional and structural characterization, although a majority of exogenously overexpressed PS polypeptides remain inactive in the low-molecular-weight complex that chiefly contains PS holoprotein, presumably because of lack of binding with other essential cofactor proteins (14). Here, we applied the baculovirus BV particle technology to the reconstitution of γ-secretase and succeeded in the selective recovery of the active form of γ-secretase, composed of fragment forms of PS1 complexed with NCT, APH-1a, and PEN-2.

MATERIALS AND METHODS

Construction of Expression Plasmid and BV Particle Preparation—Full-length cDNAs encoding PS1 in pcDNA3 vector (Invitrogen) (15) and NCT fused with V5/His tags in pEF6-V5/His vector (Invitrogen) (16) were amplified by polymerase chain reaction and inserted into pBlueBac4.5 (Invitrogen). A cDNA encoding human APH-1a was subcloned into a pEF4-myc/His A vector (Invitrogen) from a brain cDNA library (Clontech) and subsequently inserted into pBlueBac4.5. Human PEN-2 cDNA (12) was amplified from a brain cDNA library and inserted into pBlueBacHis2A (Invitrogen) in-frame with an amino-terminal His tag. D257A and D385A mutations of PS1 were inserted by the long-polymerase chain reaction method (15). Recombinant baculovirus construction, culture of Sf9 cells, and the isolation of baculovirus BV were performed as described previously (3, 4). The recombinant viruses were generated from Sf9 cells co-transfected with the vector for human PS1, NCT, APH-1a, or PEN-2 and Bac-N-Blue viral DNA (Invitrogen) by homologous recombination and sequential plaque-purification/amplification. Sf9 cells were cultured in Grace's supplemented media (Invitrogen) containing 10% fetal bovine serum, 0.1% Pluronic F-68 (Invitrogen), 100 international units/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen) in a 1-liter spinner flask at 27 °C. Sf9 cells (2×10^6 cells/ml) were co-infected with a combination of recombinant baculoviruses at a multiplicity of infection of 2 for each virus and harvested 72 h after infection. Fractionation and solubilization of Sf9 membranes in 1% CHAPSO containing HEPES buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and Complete protease inhibitor mixture (Roche Applied Sciences)) were performed as described previously (12, 17). To isolate the virus particles, the culture medium was centrifuged at $1,000 \times g$ for 15 min to precipitate cells, and the supernatant was further centrifuged at $40,000 \times g$ for 30 min. The pellets were resuspended and centrifuged again at $1,000 \times g$ for 15 min and $40,000 \times g$ for 30 min to eliminate possible contamination of cell debris. All procedures were carried out at 4 °C. The precipitates were resuspended in phosphate-buffered saline or assay buffer and stored at 4 °C until used as BV fractions. BV fractions were solubilized by 1% CHAPSO containing HEPES buffer and centrifuged. Supernatants were used as enzyme sources in *in vitro* reaction.

In Vitro γ-Secretase Assay—*In vitro* γ-secretase assay was performed as described previously with some modifications (12, 17). *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester (DAPT) was a gift from Drs. Kan and Fukuyama (University of Tokyo) (18). L-685,458 was purchased from Bachem. Membrane fractions from HeLa cells were isolated and solubilized as described previously (12, 17). Purified recombinant substrates (*i.e.* carboxyl-terminally tagged C100 or N102, respectively) were incubated with the solubilized membrane or BV fraction in modified 1× γ buffer (HEPES buffer containing 0.25% CHAPSO, 5 mM EDTA, 5 μM 1,10-phenanthroline, 10 mg/ml of phosphoramidon, and 0.1% phosphatidylcholine (Avanti)) for 6 h at 37 °C. The samples were centrifuged, and the supernatants were analyzed as described previously (12, 17).

Antibodies, Fractionation, and Immunochemical Analyses—Anti-G1Nr2 and G1L3 antibodies against glutathione *S*-transferase-fused human PS1 N terminus or hydrophilic loop, respectively, have been described previously (19, 20). Rabbit anti-PEN-2 antibody PNT3 was raised against a synthetic peptide corresponding to the amino-terminal 26 amino acids of human/mouse PEN-2. Other antibodies were purchased from Cell Signaling Technology (anti-myc 9B11, anti-cleaved Notch1 (V1744)), Invitrogen (anti-V5), or Roche Applied Sciences (anti-myc 9E10). Membrane fractionation, immunoblot analysis, immunoprecipitation, or quantitation of Aβ by two-site ELISAs were performed as described previously (12, 15–17, 19–23).

Size Exclusion Chromatography of Solubilized Sf9 Membrane and BV Fractions—1% CHAPSO-solubilized Sf9 membranes and BV fractions were diluted 4-fold (final concentration of CHAPSO, 0.25%) and loaded onto a Superose 6 HR 10/30 column (Amersham Biosciences) on an AKTA Explorer chromatography system (Amersham Biosciences). The column was eluted with HEPES buffer containing 0.25% CHAPSO. Subsequent separation of HMW Native marker kit (Amersham Biosciences) was used as a molecular weight reference. Each eluate fraction was collected and analyzed by immunoblot analysis.

RESULTS

Selective Recovery of PS1 Fragments and Cofactor Proteins on BV—We infected Sf9 cells with a recombinant baculovirus containing a cDNA encoding human PS1 that harbors the catalytic site of γ-secretase. Previous studies showed that endoproteolysis of PS is associated with the acquisition of γ-secretase activity in mammalian cells (5–8). Immunoblot analysis of Sf9 membrane fractions showed a robust expression of PS1 polypeptide chiefly in a holoprotein form (Fig. 2*A*, *left lane*), which is known to be γ-inactive and which was in agreement with previous reports (24, 25).

We next infected the Sf9 cells with various combinations of recombinant baculoviruses encoding other γ-secretase components (*i.e.* NCT, APH-1a, and PEN-2, collectively referred to as cofactors) in addition to PS1. Immunoblot analysis showed that PS1 underwent endoproteolysis to generate an amino-terminal fragment and a carboxyl-terminal fragment corresponding to the size of mammalian PS1 fragments only when all four components were infected, although a major proportion of recombinant PS1 polypeptide remained as a holoprotein (Fig. 2*A*, *right lane*). In contrast, PS1 fragments were hardly generated upon expression of PS1 plus one or two of the cofactor proteins (Fig. 2*A* and data not shown). Co-immunoprecipitation studies revealed that any two of the components among PS1 (holoprotein as well as fragments), NCT, APH-1a, or PEN-2 were co-immunoprecipitated in Sf9 membranes that expressed the four components (data not shown).

We then analyzed the BV fractions from Sf9 cells infected with combinations of the four components by immunoblotting. In contrast to Sf9 cell membranes, PS1 polypeptides accumulated almost exclusively in the fragment forms, together with NCT, APH-1a, and PEN-2 in the BV fractions, only when all four components were infected (Fig. 2*A*, *right lane*). Co-immu-

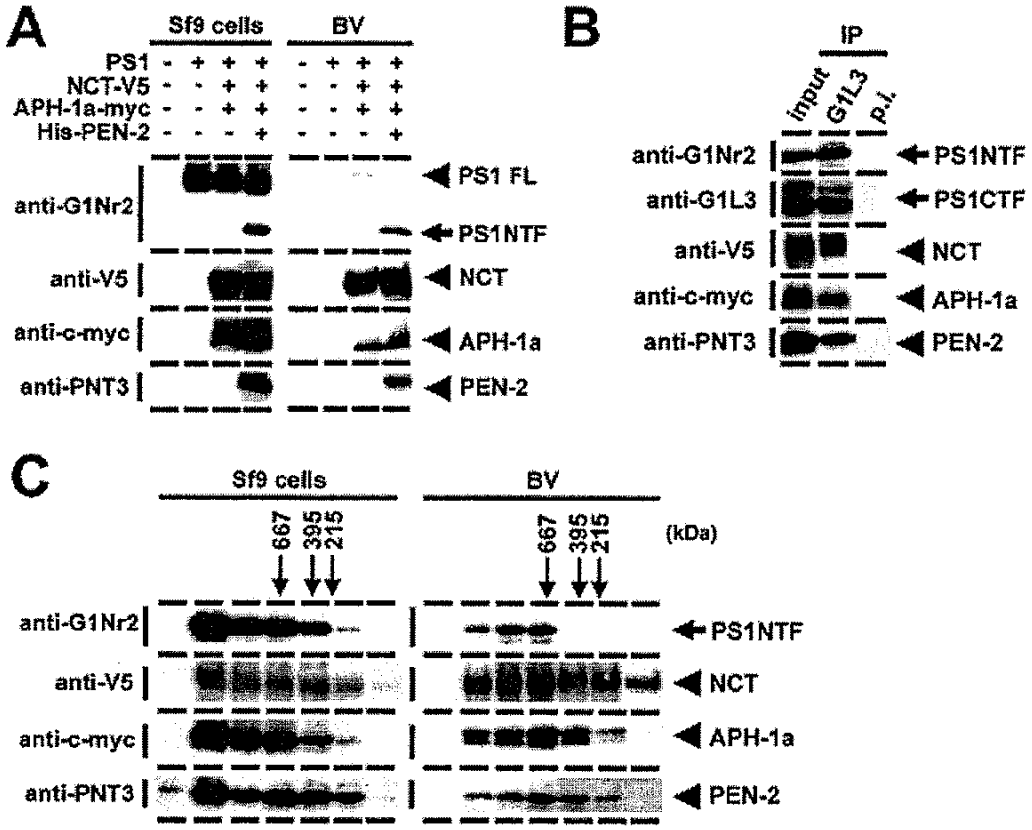

FIG. 2. **Characterization of reconstituted PS1 complex.** *A*, immunoblot analysis of Sf9 membranes infected with combinations of PS1, NCT-V5/His, APH-1a-myc/His, His-PEN-2, or BV particles. Note that PS1 fragments (*arrowheads*) were detected in lysates from cells or BV thereof infected with all four components. *B*, co-immunoprecipitation (*IP*) analysis of solubilized BV. Immunoprecipitates with anti-PS1 antibody (*G1L3*) or preimmune serum (*p.i.*) were analyzed by Western blotting with antibodies to PS1 or other components. *C*, separation of reconstituted PS1 complex in BV by gel filtration.

noprecipitation studies revealed that amino-terminal fragment and carboxyl-terminal fragment of PS1 were associated as a heterodimer and formed a protein complex with NCT, APH-1, and PEN-2 in a similar manner to that in mammalian cells (Fig. 2*B*). We next fractionated the Sf9 membranes and BV particles solubilized in CHAPSO by size-exclusion chromatography in 0.25% CHAPSO containing buffer, a condition in which solubilized membranes exhibit γ-secretase activity. Under this condition, PS1 fragments and γ-secretase activities in HeLa membranes were separated in fractions >1 megadalton, as described previously (26).[2] Immunoblot analysis of the eluates of Sf9 cells and BV revealed that fragment forms of PS1 as well as other components were predominantly separated in fractions of HMW ranges >1 megadalton (Fig. 2*C*). Similar results were obtained by glycerol velocity centrifugation (Fig. 1S). These data suggest that recombinant γ-secretase components undergo complex formation in Sf9 cells infected by baculovirus in a similar manner to that in mammalian cells, and that the HMW complex associated with PS1 fragments, but not a holoprotein, is selectively accumulated on BV particles.

Characterization of Reconstituted γ-Secretase Activity in Vitro in Sf9 Cell Membranes or on BV—To characterize the endogenous and reconstituted γ-secretase activities in Sf9 cells, we solubilized the Sf9 membranes with 1% CHAPSO and incubated them with the recombinant C100 substrate *in vitro* (12, 17). We did not detect any *de novo* generation of Aβ peptides from the recombinant substrate in mock-infected Sf9 membranes, even in the presence of phosphatidylcholine (Fig. 3*A*, *left*), which increases *in vitro* γ-secretase activity in solubilized mammalian membranes (27).[3] Some γ_{42}-secretase activity was observed, but this activity was not sensitive for γ-secretase inhibitors (see below). This was in agreement with the observation that Sf9 cells do not harbor an endogenous γ-secretase activity (24, 25). Overexpression of combinations of any three components did not reconstitute γ-secretase activities to elicit *de novo* Aβ generation *in vitro* (data not shown), whereas overexpression of all four components exhibited a dramatic up-regulation of the proteolytic activity in Sf9 cell membranes (Fig 3*A*). Interestingly, Aβ42 was the predominantly generated Aβ species relative to Aβ40 when we used Sf9 cells as an enzyme source, and the percentage of Aβ42 as a fraction of *de novo* generated total Aβ amounted to ~60%. We next analyzed the γ-secretase activity in CHAPSO-solubilized BV particles from multiply infected Sf9 cells and observed a robust Aβ-generating activity in the BV fraction from cells infected with all four components. These activities were ~10-fold higher than that detected in mammalian HeLa cell membranes, the latter harboring the highest level of endogenous γ-secretase activity in mammalian cells (Fig. 3*B*) (17). Similarly to the results in Sf9 membranes, Aβ42 was again the predominant species in *de novo* generated Aβ (%Aβ42: mean ± S.E., 65.5 ± 7.1%). Coincubation with γ-secretase inhibitors, L-685,458 or DAPT, abolished the activity reconstituted in BV fractions (Fig. 3*C*). Notably, reconstituted γ_{42}-secretase activity was less sensitive to L-685,458 at 100 nM concentration (65.2% inhibition), which completely abolished the γ-secretase activity in the

[2] I. Hayashi, Y. Urano, R. Fukuda, N. Isoo, T. Kodama, T. Hamakubo, T. Tomita, and T. Iwatsubo, unpublished data.

[3] I. Hayashi, Y. Urano, R. Fukuda, N. Isoo, T. Kodama, T. Hamakubo, T. Tomita, and T. Iwatsubo, unpublished observations.

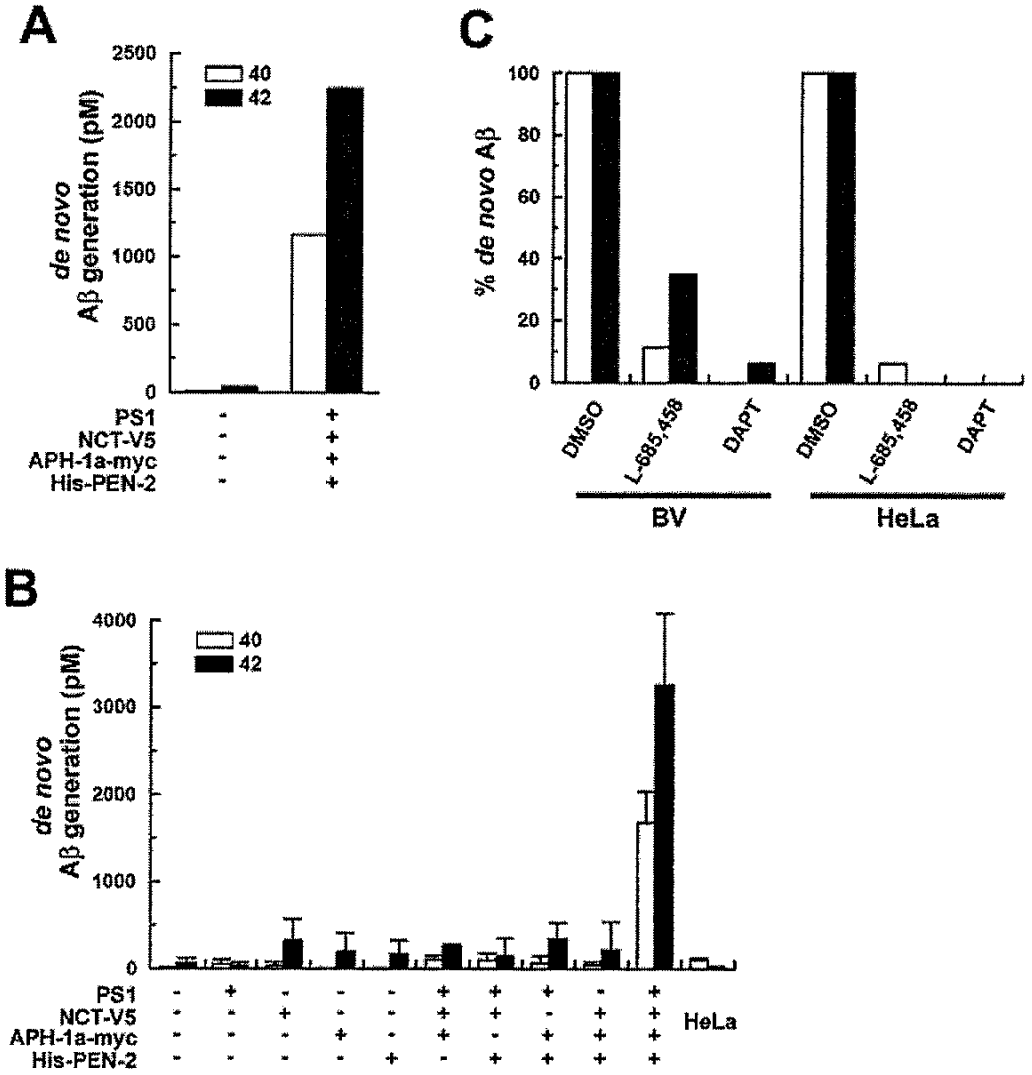

FIG. 3. **Characterization of reconstituted γ-secretase activity.** *A*, levels of *de novo* generated Aβ40 (□) and Aβ42 (■) ($n = 2$) generated from recombinant C100 by *in vitro* coincubation with solubilized Sf9 membranes infected with PS1, NCT-V5/His, APH-1a-myc/His, or His-PEN-2. *B*, levels of Aβ ($n = 3$, mean ± S.E.) generated from recombinant C100 by *in vitro* coincubation with solubilized BV particles infected with various combinations of γ-secretase components. HeLa cell membrane fraction was used as a control. *C*, effect of γ-secretase inhibitors. Recombinant substrates were coincubated with solubilized BV or HeLa membrane in the presence of DAPT (100 μM) or L-685,458 (100 nM). Both inhibitors inhibited the reconstituted γ-secretase activity.

HeLa membrane, whereas DAPT potently inhibited the γ_{42}-secretase activity (94.0% inhibition; residual activity was almost equivalent to that in mock-infected BV). These results indicate that γ-secretase activity can be reconstituted in Sf9 cells and BV associated with robust γ_{42}-cleavage activity by overexpression of all four components of human γ-secretase, as shown previously in other organisms (9–13).

We then examined the γ-secretase activity reconstituted by infection of mutant PS1 carrying D257A or D385A mutations, which is known to be inactive for PS1 endoproteolysis as well as for γ-secretase activity (28), together with the three cofactor proteins. Recombinant PS1/D257A as well as PS1/D385A did not undergo endoproteolysis in Sf9 cells as well as in BV fractions (Fig. 4*A*), although they formed a >1-megadalton protein complex with other components (Fig. 4*B*). Reconstituted γ-secretase complex containing recombinant PS1/D257A or PS1/D385A showed almost no Aβ-generating activity, whereas a low level of residual γ_{42}-secretase activity that was almost comparable with that in mock-infected BV was observed (Fig. 4*C*). The identity of this endogenous, γ-secretase-like enzymatic activity should be examined further. Thus, intramembranous aspartates are required for endoproteolysis as well as for the Aβ-generating enzymatic activity in a reconstituted γ-secretase complex in an insect cell system, similarly to that seen in mammalian cells (28).

γ-Secretase cleaves several type I membrane proteins other than βAPP, including Notch, at multiple sites within the transmembrane domain and liberate the ICD (7). To examine whether reconstituted γ-secretase activity in BV cleaves other substrates, we examined the ICD generation from recombinant βAPP or Notch carboxyl-terminal fragments (C100 and N102, respectively (17)) using the BV particles as the source of γ-secretase *in vitro*. The recombinant substrates were cleaved to generate ICDs by the γ-secretase activity from BV fractions of Sf9 cells infected with all four components, which were sensitive to γ-secretase inhibitors, in a similar manner to those with HeLa-derived γ-secretase (Fig. 5). Finally, we compared the γ-secretase activities in solubilized BV particles and in Sf9 membranes. When normalized by the expression levels of the PS1 amino-terminal fragment, the relative γ-secretase activity in BV particles was increased by ~2.5 fold compared with that of solubilized Sf9 cell membranes, suggesting that the γ-secretase active form of PS1 fragments was enriched in BV fractions (Fig. 6). Thus, we were able to reconstitute the γ-secretase

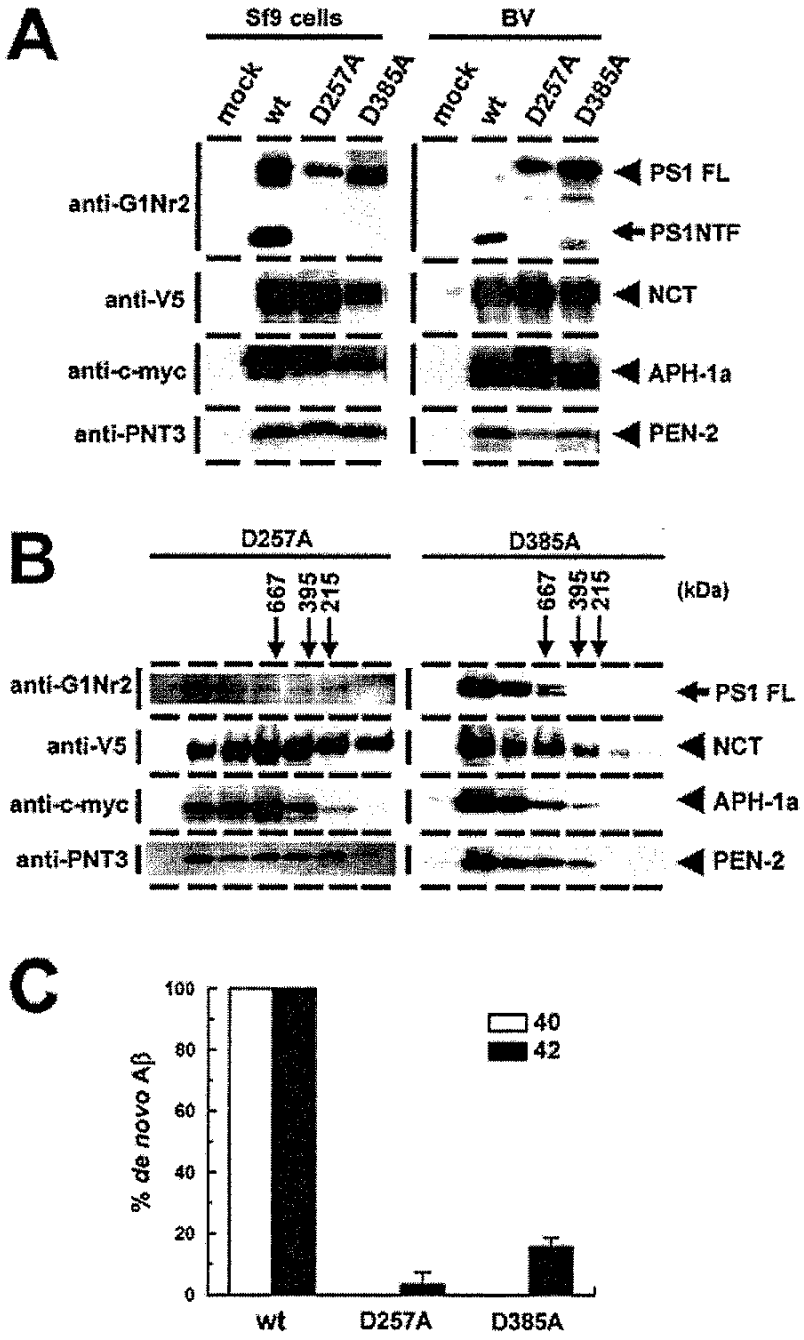

FIG. 4. **Characterization of reconstituted γ-secretase complex containing PS1 harboring aspartate mutations.** *A*, immunoblot analysis of Sf9 membranes expressing aspartate mutant PS1 together with NCT-V5/His, APH-1a-myc/His, His-PEN-2, or the BV particles. Note that PS1 fragments (*arrowhead*) were not detected in cell lysates or BV thereof infected with recombinant cDNA encoding aspartate mutant PS1. *wt*, wild type. *B*, separation of mutant γ-secretase complex by gel filtration. Note that mutant PS1 holoproteins were detected within fractions with molecular masses over 1 megadalton. *C*, γ-secretase activity of reconstituted aspartate-mutant γ-secretase complex ($n = 3$, mean ± S.E.). Mutation in either of the aspartates virtually abolished the γ-secretase activity *in vitro*. *wt*, wild type.

complex in BV membranes. This complex contained the fragment forms of PS1 and was enriched in proteolytic activity.

DISCUSSION

In this study, we selectively reconstituted and recovered human γ-secretase complex in an active form, using BV particles. The baculovirus/insect cell system is one of the most widely used methodologies for overexpression of recombinant proteins, including multispan integral membrane proteins (reviewed by Massotte (1)). We used this system for the reconstitution of γ-secretase, a pivotal enzyme for the generation of Aβ peptides in Alzheimer's disease brains. We found that γ-secretase complex is reconstituted in membrane fractions of Sf9 cells infected with PS1, NCT, APH-1, and PEN-2 in an active state, showing similar metabolic and enzymatic properties to those in mammalians cells. The requirement of the expression of all four components for the reconstitution of γ-secretase complex and its activity, which was demonstrated previously in yeast (13), suggests that Sf9 cells do not harbor endogenous cofactor proteins that can efficiently form complexes with the exogenous human PS1. Even with the co-infection of all four components, however, a majority of exogenous PS1 remained in a holoprotein form, and relatively small amounts of PS1 fragments were generated in Sf9 membranes, similarly to overexpression systems using other organisms, including yeast (9–13).

Recent reports that G-protein-coupled receptors can be reconstituted in a functional form, recruiting endogenous Gα subunit or adenylyl cyclase as interactors in the extracellular BV particles released from infected Sf9 cells (2), prompted us to apply this strategy to the effective reconstitution of γ-secretase composed of multiple integral membrane proteins. Surprisingly, BV fractions from Sf9 cells infected with all four components almost exclusively contained the γ-secretase complex harboring PS1 fragments, with minimal contamination of nascent or misfolded PS1 holoproteins. Reconstituted γ-secretase

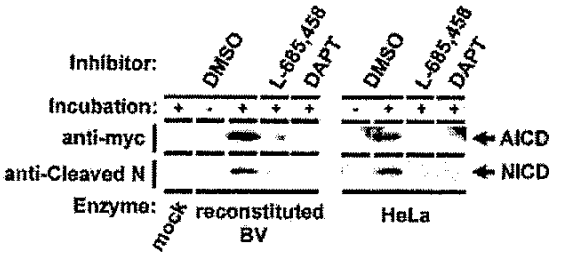

FIG. 5. **Reconstituted γ-secretase activities for ICD generation.** Recombinant substrates were incubated at 4 °C (−) or 37 °C (+) with solubilized membrane fractions from mock-infected BV (*mock*), BV infected with all four components (*reconstituted BV*), or HeLa cell membrane in the presence of dimethyl sulfoxide (*DMSO*), DAPT (100 μM), or L-685,458 (100 nM), respectively. Resultant samples were analyzed by immunoblotting using anti-myc (βAPP) or anti-cleaved Notch1 (Val1744) antibodies. *AICD*, βAPP ICD; *NICD*, Notch ICD.

on BV again reproduced similar biochemical and enzymatic properties to those in mammalian cells (*i.e.* HMW protein complex formation, inhibition by γ-secretase inhibitors, requirement of the transmembrane aspartates, and cleavage of both βAPP and Notch). The mechanism whereby reconstituted γ-secretase in Sf9 cells or BV shifted the *de novo* generation of Aβ to Aβ42 species remains unclear, but it is highly reminiscent of the overproduction of Aβ42 by expression of wild-type *Drosophila* PS in mammalian cells, which predominantly generates Aβ40 in *Drosophila* cells (23); the three-dimensional structure of the catalytic pocket of γ-secretase, which determines the positions of the preferred γ-secretase cleavage site, may be altered depending on the cell species or the environment surrounding the enzyme complex. Moreover, very high γ-secretase activity (~10 fold that of mammalian HeLa cells) was recovered in BV. Notably, the relative γ-secretase activity standardized by the levels of PS1 fragments also was higher than that in the Sf9 cell membrane. This supports the notion that a limited proportion of HMW protein complex composed of the four components is γ-secretase-active, as suggested by the limited recovery of active γ-secretase complex using activity-dependent purification (29, 30), and that some additional factors or conditions are necessary for the full activation of γ-secretase (31).

The mechanism whereby "active form" γ-secretase complex containing PS1 fragments is selectively accumulated on BV is unknown. One possibility would be that the BV envelope may preferentially recruit membranes derived from plasma membrane or late intracellular membrane compartments, which are enriched in an active form of γ-secretase (32–34). However, the observation that endoplasmic reticulum-resident membrane proteins, such as SREBP or SREBP cleavage-activating protein, can be recovered on BV as well (4) may argue against this view. Folding, complex formation, and functions of integral membrane proteins may be properly achieved on BV, based on some unknown mechanism specific to the life cycle of baculovirus. Intriguingly, the major phospholipid consisting of BV envelope is phosphatidylserine, whereas phosphatidylcholine and phosphatidylethanolamine are the major phospholipids in host Sf9 cells (35–37). Recently, palmitoylation of GP64 protein, which is highly abundant and necessary for efficient budding and production of BV as well as its entry into host cells, was reported (38). However, GP64 was not detected in lipid rafts in Sf9 cells, and its palmitoylation did not affect the apparent exclusion of GP64 from lipid rafts, although lipid rafts have been shown to play roles in the infection cycles of several viruses (*e.g.* HIV-1 and influenza virus) (39). These results may suggest that specific membrane domains other than rafts may be involved in baculovirus budding as well as in the effective formation of a functional protein complex in Sf9 cells.

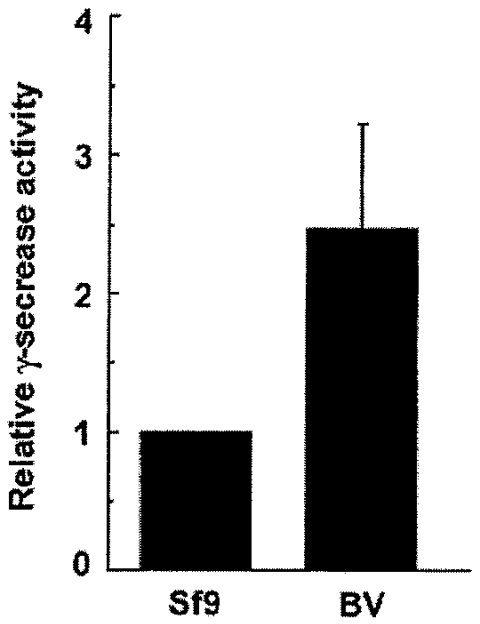

FIG. 6. **Comparison of γ-secretase activities in solubilized Sf9 cell membranes (*Sf9*) and BV particles (*BV*).** Relative γ-secretase activities (that of Sf9 cell membrane = 1) were evaluated by the amount of Aβ1–40 generated from recombinant C100 (as measured by ELISA) and normalized by the levels of PS1 amino-terminal fragment (as quantified by densitometric analysis (n = 3, mean ± S.E.)).

We and others have reconstituted the γ-secretase complex by overexpression of the four components in mammalian, *Drosophila*, or yeast cells (10–13). However, the enzymatic activity in BV was by far higher than those in mammalian or *Drosophila* cells (Fig. 3*B* and data not shown). Considering the previous observations on the effective incorporation of endogenous proteins into the functional protein complex (*e.g.* exogenous G-protein coupled receptor and endogenous G protein) observed in BV (2), it is possible that the γ-secretase complex reconstituted in BV incorporated relevant binding partner proteins that were endogenously present in BV, resulting in the formation of a "hyperactive" enzyme complex. Approximately 30 proteins have been identified by SDS-PAGE and Coomassie blue staining in the BV envelope (35). Recently, proteomic analysis of the occlusion-derived form of baculovirus was reported, although similar data on BV have not been publicized yet (40). Thus, it would be possible to identify the missing interacting partners of the PS complex in Sf9 proteins based on proteomic analysis of the γ-secretase reconstituted on BV. Other possible applications of the reconstituted γ-secretase on BV may include production of active form-specific or blocking antibodies against γ-secretase and generation of an immobilized γ-secretase as a biochip sensor that may be applicable to large-scale screening of physiological interactors or low-molecular-weight compounds for drug discovery (4).[4]

A number of integral membrane proteins with important functions, including ion channels or receptor proteins, form hetero-multimeric protein complexes (1). Purification of a large quantity of protein complex in a biologically active state is indispensable to the biochemical, functional, and especially structural studies of these complexes. The purification procedures presented in this report for recombinant membrane proteins in an active form in BV fractions, recovered from multiply infected Sf9 cells, should provide a versatile methodology for the characterization of various integral membrane protein

[4] T. Hamakubo, Y. Urano, R. Fukuda, and T. Kodama, unpublished result.

complexes. It should also open up the way to one of the ultimate goals in γ-secretase research—to crystallize the complex and unravel its three-dimensional structure in an active state.

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EXHIBIT B

US006713278B1

(12) **United States Patent**
Bouvier et al.

(10) **Patent No.: US 6,713,278 B1**
(45) **Date of Patent: Mar. 30, 2004**

(54) **PREPARING MEMBRANE RECEPTORS FROM EXTRACELLULAR BACULOVIRUSES**

(75) Inventors: **Michel Bouvier**, Montréal (CA); **Thomas Loisel**, Montréal (CA); **Stefano Marullo**, Paris (FR); **Pierre Boulanger**, Montpellier (FR); **Arthur Donny Strosberg**, Paris (FR)

(73) Assignees: **Valorisation-Recherche, Limited Partnership**, Montreal (CA); **Centre National de la Recherche Scientifiquê**, Paris Cedex (FR)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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(22) PCT Filed: **Apr. 10, 1998**

(86) PCT No.: **PCT/FR98/00736**

§ 371 (c)(1),
(2), (4) Date: **Feb. 29, 2000**

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PCT Pub. Date: **Oct. 22, 1998**

(30) **Foreign Application Priority Data**

Apr. 11, 1997 (FR) 97 04476

(51) **Int. Cl.**[7] **C12P 21/06**

(52) **U.S. Cl.** **435/69.1**; 435/5; 435/252.3; 435/320.1; 536/23.8

(58) **Field of Search** 435/69.1, 5, 252.3, 435/320.1; 536/23.2

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Parker et al. Reconstitutively Active G Protein–coupled Receptors Purified from Baculovirus–infected Insect Cells. J. Biol. Chem. 266(1):519–526, Jan. 1991.*

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Primary Examiner—John Ulm
(74) *Attorney, Agent, or Firm*—Nixon & Vanderhye

(57) **ABSTRACT**

Method for producing membrane receptors based on a baculovirus/insect cell system. The invention further includes the receptors produced by the method described herein, a model for studying the properties of these membrane receptors and an assay to screen molecules that are active on these membrane receptors.

5 Claims, 5 Drawing Sheets

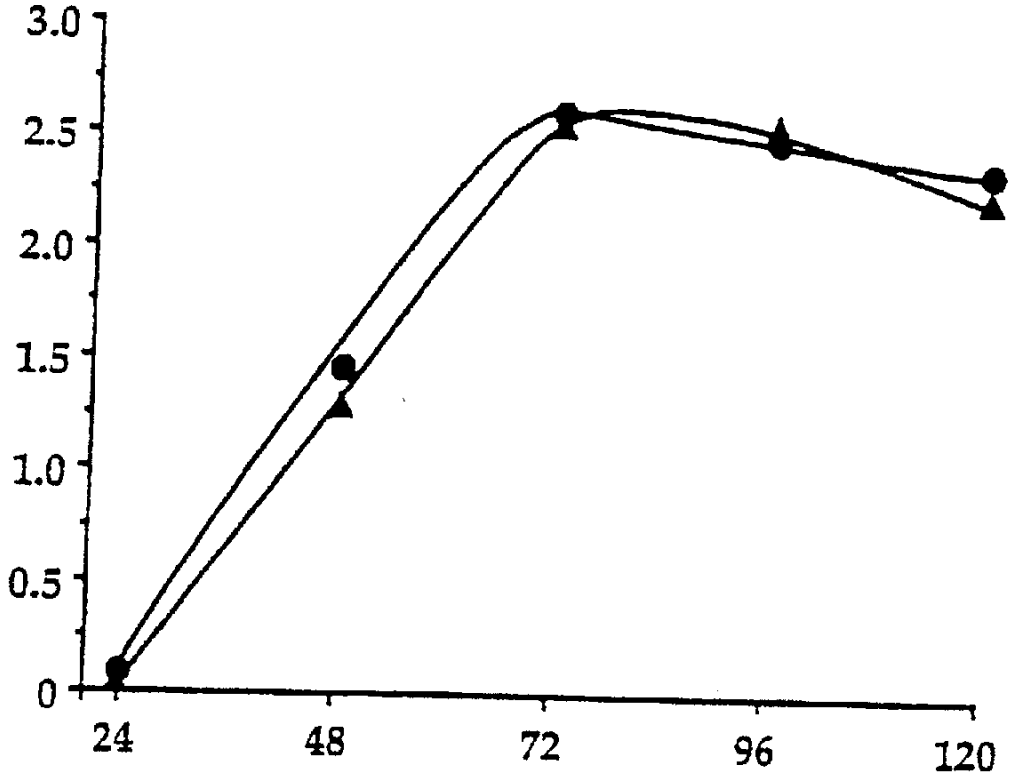

figure 1

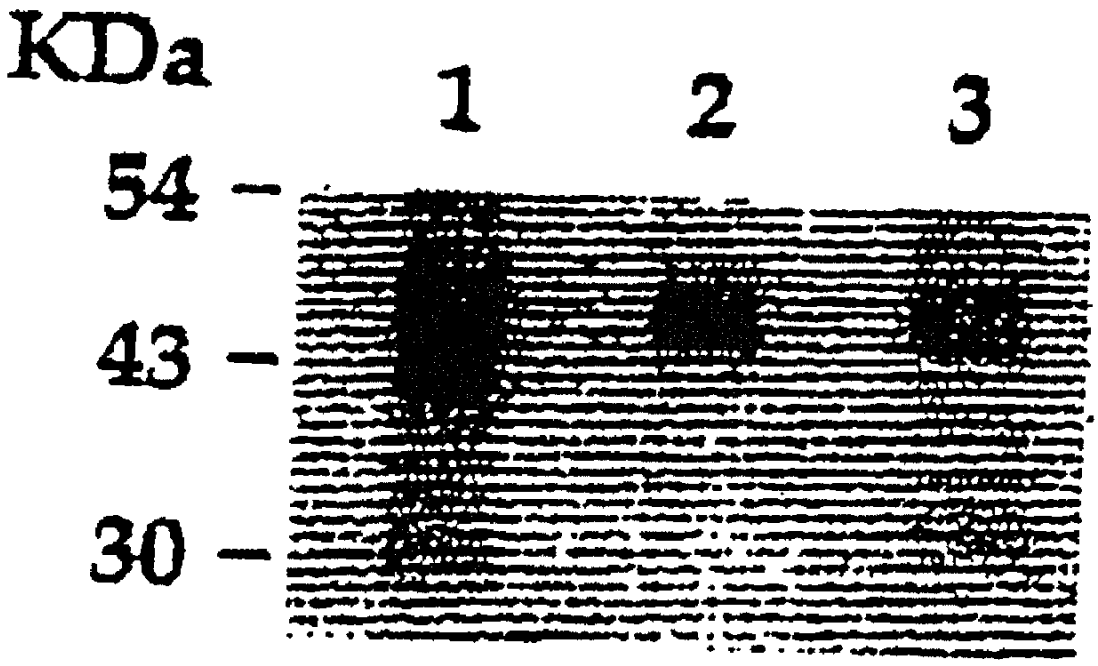

Figure 2

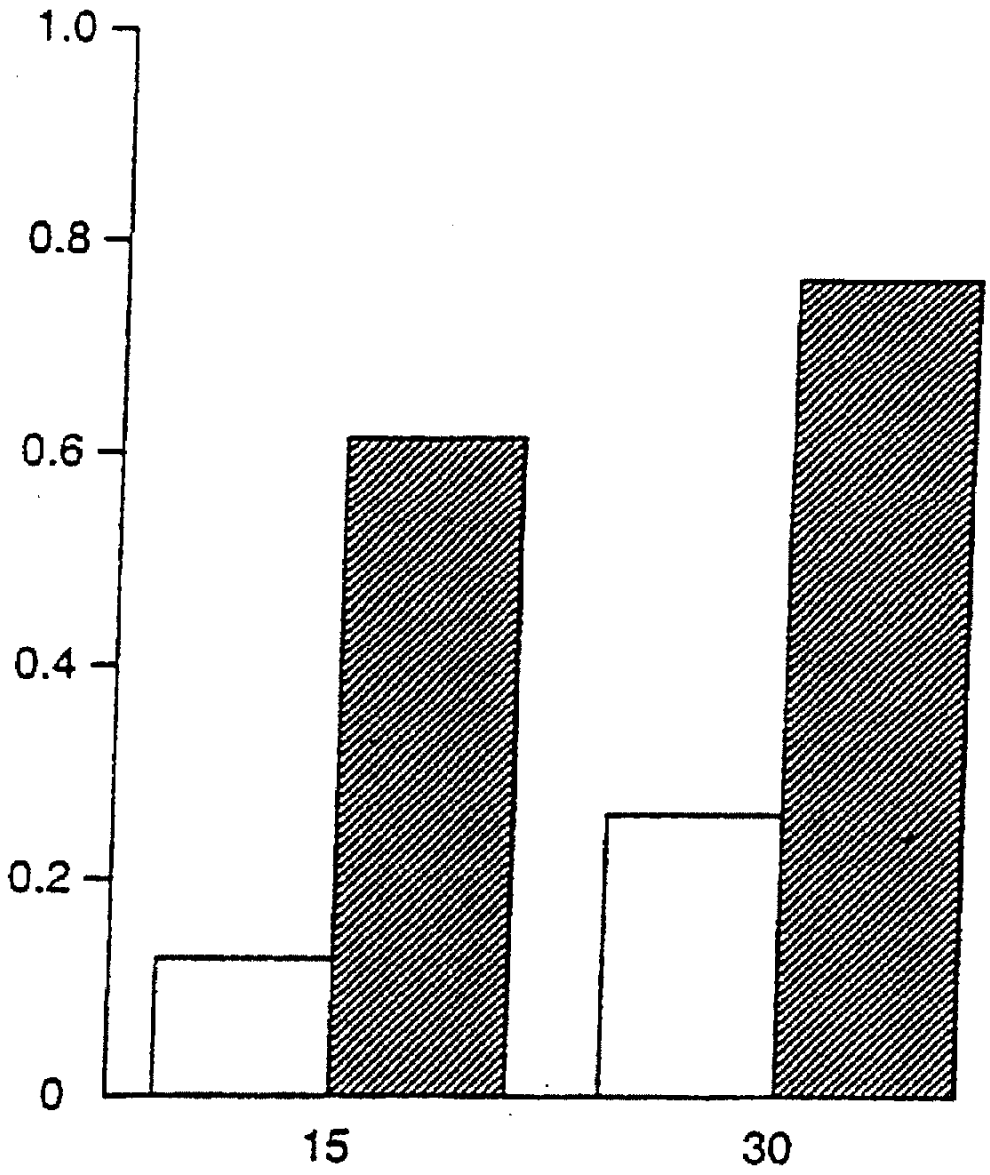

Figure 3

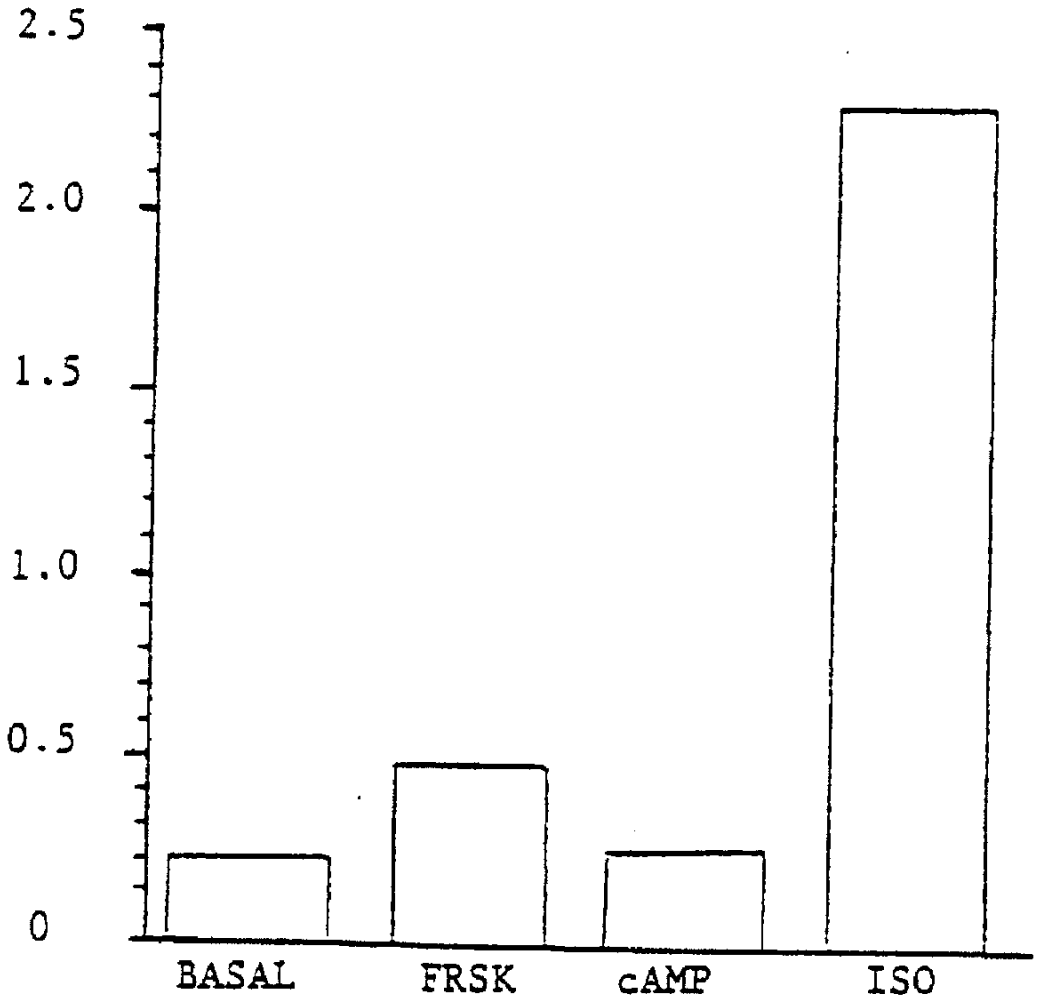

FIGURE 4

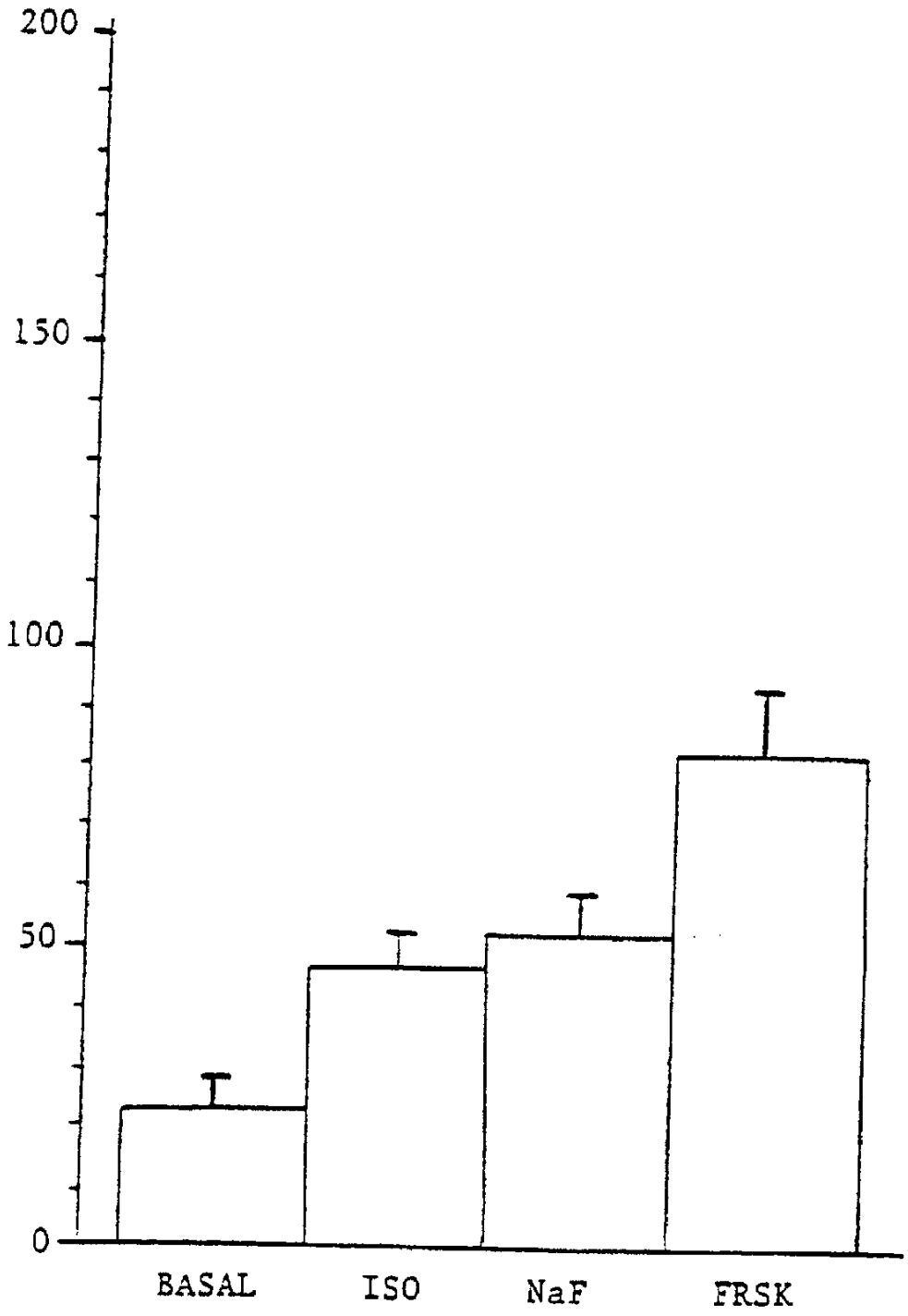

FIGURE 5

PREPARING MEMBRANE RECEPTORS FROM EXTRACELLULAR BACULOVIRUSES

FIELD OF THE INVENTION

The invention relates to the production of membrane receptors in a baculovirus/insect cell system.

BACKGROUND OF THE INVENTION

Over the past few years, heterologous expression systems have often been used to study the expression as well as the pharmacological and biochemical characteristics of membrane receptors.

Although a significant expression can be obtained in some expression systems in mammalian cells, there have been problems, in particular in the case of some types of receptors such as the G-protein-coupled receptors.

The G-protein-coupled receptors belong to the superfamily of receptors with seven transmembrane domains. They comprise, for example, the adrenergic or muscarinic receptors, and all have the same structure which is made up of a polypeptide chain comprising seven hydrophobic domains which cross the membrane lipid bilayer.

When it is desired to express these receptors in mammalian cell systems, a relatively low density of receptors expressed by the said cells, rarely exceeding a few picomoles of receptor per milligram of membrane protein, is generally obtained. Although these levels of expression are sufficient for a functional and pharmacological characterization, they clearly limit the type of biochemical, biophysical and structural studies which can be carried out. A fortiori, this expression system cannot be used for the production of receptors in a large quantity, for example for their therapeutic use.

To increase the quantity of receptors obtained, various teams have sought to produce them in a baculovirus/insect cell system; in many cases, baculoviruses expressing G-protein-coupled receptors have been able to produce these recombinant receptors in cells of the *Spodoptera frugiperda* Sf9 or Sf21 lines, up to levels reaching 30 to 100 picomoles per milligram of membrane protein. These systems have made it possible to make significant progress in the study of the palmitoylation of receptors and also to study the effects produced by various agonists and antagonists, or to carry out the reconstitution of artificial receptors.

However, the baculovirus/insect cell system has the major disadvantage of expressing a high proportion of inactive receptors. The receptors, which are recovered in the membrane fraction of the cells infected with the baculoviruses are in an immature and incompletely glycosylated form. This probably results from a saturation of the normal post-translational maturation pathway, which brings about the retention of immature receptors in the membranes of the endoplasmic reticulum or in the Golgi apparatus. To obtain functional receptors, it is necessary in this case to include a purification step based on the biological activity of the receptor (for example an affinity chromatography step).

It would therefore be necessary to develop a system which makes it possible to easily separate the plasma membrane comprising the mature receptors from the other membrane fractions such as the endoplasmic reticulum or the membranes of the Golgi apparatus which comprise the biologically inactive, immature receptor.

SUMMARY OF THE INVENTION

It has recently been shown that the infection of Sf9 cells with a baculovirus encoding the HIV1 Gag gene (Pr55 Gag) brings about the budding of particles carrying the Gag protein (Gag particles) which are released into the extracellular medium. It has been suggested that these Gag particles carry with them, during their budding, the plasma membrane and the proteins associated with it.

The inventors have formulated the hypothesis that the coexpression, in a baculovirus/insect cell system, of a G-protein-coupled receptor and of Pr55 Gag can promote the release of the Gag particles expressing only the mature receptors which are correctly inserted into the plasma membrane. To test this hypothesis, the inventors infected Sf9 cells with baculoviruses encoding the human adrenergic receptor β2AR and the Pr55 Gag protein. Surprisingly, they then observed that the β2AR receptor is almost completely absent from the Gag particles, but is, on the other hand, present at a high density in extracellular baculovirus particles. In addition, the receptors expressed in these extracellular baculoviruses are correctly glycosylated and normally active.

The present invention relates to the use of these extracellular baculoviruses for the production of preparations of a membrane receptor.

The subject of the present invention is a method of producing a recombinant membrane receptor in a baculovirus/insect cell system, from a culture of insect cells infected with a recombinant baculovirus expressing the gene encoding the membrane receptor, which method is characterized in that the membrane receptor is obtained from extracellular baculoviruses produced by the infected cells.

According to a preferred embodiment of the present invention, the receptor belongs to the superfamily of receptors with seven transmembrane domains; this is for example a receptor of the family of G-protein-coupled receptors.

Recombinant baculoviruses expressing the gene encoding the membrane receptor which it is desired to produce are obtained by cloning the gene under transcriptional control of an appropriate promoter of the baculovirus, according to methods well known per se to persons skilled in the art.

Any strong baculovirus promoter which can be used for the expression of heterologous genes, such as for example the polyhedrin promoter (polh) or that of the P10 protein, may be used for the production of a recombinant baculovirus which can be used within the framework of the present invention.

According to a preferred embodiment of the method in accordance with the invention, it comprises a step during which the extracellular baculoviruses produced by the infected cells are harvested and they are separated from the cellular fractions. The harvesting and the separation of the extracellular baculoviruses may be carried out by successive centrifugations, for example in the following manner: a first centrifugation is carried out at about 500×g, at the end of which the supernatant containing the extracellular baculoviruses is collected. This supernatant is subjected to a centrifugation at about 45,000×g; the resulting pellet which contains the extracellular baculoviruses is resuspended, and the suspension is subjected to a centrifugation at about 500×g; the supernatant resulting from this centrifugation is centrifuged at about 45,000×g, and the pellet, which contains the extracellular baculoviruses, is recovered. Advantageously, the extracellular baculoviruses may also be purified by sucrose gradient centrifugation, or any other equivalent method.

According to another preferred embodiment of the method in accordance with the invention, it comprises a step during which the extracellular baculoviruses produced by

the infected cells are lysed; advantageously, it also comprises a step during which the lysate obtained at the end of the preceding step is fractionated, and the fraction comprising the membrane receptor is recovered.

The purified preparations and the lysates of extracellular baculoviruses, as well as their fractions comprising the membrane receptor, which are capable of being obtained by the methods defined above constitute membrane receptor preparations which also form part of the subject of the present invention. These preparations consist of active and fully mature receptors, unlike the membrane receptor preparations obtained in the prior art from the plasma membranes of infected cells, which comprise a high proportion of inactive receptors, and which can only be used after an additional step of purification on the basis of the activity of the relevant receptors, for example after affinity chromatography.

By contrast, the membrane receptor preparations in accordance with the invention are characterized in that, prior to any purification carried out on the basis of the activity of the relevant receptor, at least 90%, and preferably at least 95%, of the receptor is in an active form.

Preparations, in accordance with the invention, of a membrane receptor may be used to prepare the receptor in a purified form, with a much better yield than that which could be achieved from membrane receptor preparations obtained in the prior art from the plasma membranes of infected cells.

The membrane receptor preparations in accordance with the invention, as well as the extracellular baculoviruses obtained using the method in accordance with the invention, may also be used directly, for example as a system for studying the properties of membrane receptors, as a system for screening molecules which are active on these membrane receptors, or alternatively for studying their post-translational modifications such as phosphorylation or palmitoylation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: WAR activity in extracellular recombinant baculoviruses.

FIG. 2: Comparison of the forms of β2AR receptor present in cell membrane preparations and in extracellular baculoviruses.

FIG. 3: Palmitoylation of β2AR receptors expressed in extracellular baculoviruses. Clear bars: incorporation in the absence of isoproterenol; Etched bars: incorporation in the presence of isoproterenol.

FIG. 4: Comparison of forms of the β2AR receptor. On the y-axis: relative incorporation of ^{32}P (arbitrary units). On the x-axis: BASAL=control; FRSK=incorporation in the presence of forskolin; cAMP=incorporation in the presence of dibutyril cyclic AMP; and ISO=incorporation in the presence of isoproterenol.

FIG. 5: Functionality of the β2AR receptor expressed in extracellular baculoviruses. On the y-axis: adenyl cyclase activity (in picomoles of cyclic AMP/min/mg of protein). On the x-axis: BASAL=control; FRSK=incorporation in the presence of forskolin; NaF=incorporation in the presence of NaF; and ISO=incorporation in the presence of isoproterenol.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be understood more clearly with the aid of the additional description which follows, which refers to exemplary embodiments of the method in accordance with the invention for the preparation of membrane receptors.

EXAMPLE 1

Preparation of Recombinant Baculoviruses Expressing a G-Protein-Coupled Receptor

A recombinant baculovirus expressing β2AR is obtained by cloning a DNA sequence consisting of the cDNA for β2AR in fusion with the c-myc epitope, obtained as described by MOUILLAC et al. [J. Biol. Chem., 267, 21733–21737 (1992)], at the NheI site of the transfection/recombination vector pJVNheI (marketed by the company INVITROGEN). This vector was transfected with the linearized genome of a baculovirus AcMNPV (sold by the company INVITROGEN) into Sf9 cells; the recombinant baculovirus obtained in this manner is called c-myc-β2AR.

In the same manner, the sequence encoding the muscarinic receptor M1 and the sequence encoding the dopaminergic receptor D1 [respectively described by ALLARD et al. Nucleic Acid Research, 15, p 10604, (1987) and by DEARRY et al., Nature, 347, p 72, (1990)] were cloned in order to obtain the recombinant baculoviruses (respectively called M1-R and D1-R) expressing these receptors.

a) Culture and infection of the cells, and harvesting of the extracellular baculoviruses:

Sf9 cells are cultured at 27° C. in culture flasks in a 100 ml suspension (BELLCO GLASS) in supplemented GRACE medium (GIBCO) containing 10% foetal calf serum (FCS), and 0.001% pluronic acid. 60 ml of suspension of cells (2×10^6/ml) are infected with the recombinant baculovirus expressing P2AR, D1 or M1, at a multiplicity of infection varying between 2 and 5.

The cells are harvested by centrifugation at 500×g for 5 min at 4° C.

The viral particles are isolated after harvesting the cells, by centrifugation of the culture supernatant at 45,000×g for 20 min at 4° C. The pellets obtained are resuspended at 4° C. in a volume of phosphate-buffered saline (PBS) equal to ½oth of the volume of the initial culture, and centrifuged at 500×g for 5 minutes at 4° C.; the supernatant for this centrifugation at 500×g is again centrifuged at 45,000×g for 20 min at 4° C.

b) Purification of the baculovirus particles on a sucrose gradient

The pellet of viral particles which is obtained from 100 ml of cultures of Sf9 cells infected with the recombinant baculovirus expressing β2AR, M1 or D1 is resuspended in 1.2 ml of TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), in the presence of protease inhibitors).

The suspension is deposited at the top of a tube containing a linear gradient (25%–56%) of sucrose in TE solution. The tubes are centrifuged at 100,000×g for 90 minutes. The gradient is collected from the top to the bottom of the tube, in 20 fractions. The first fraction has a volume of 1.4 ml, and the other 19 are 500 μl.

EXAMPLE 2

Demonstration of the β2AR Activity in the Extracellular Recombinant Baculoviruses

The Sf9 cells infected with the recombinant baculovirus expressing β2AR are cultured, and the baculoviruses are harvested as described in Example 1 a), 24 hours, 48 hours, 72 hours, 96 hours and 120 hours after the infection.

The activity of the β2AR receptors is evaluated by measurements of saturation and of competitive binding which are carried out as described by BOUVIER et al. [Mol. Pharmacol. 33 :133–139 (1982)] using [^{125}I] iodocyanopindolol ([^{125}I]ICYP) as labelled ligand.

The pellets of viral particles which are obtained are resuspended at 4° C. in the buffer which will be used for the reaction. Aliquots of this suspension of viral particles, corresponding to 0.2 to 1 μg of proteins are mixed with 5 to 350 pM of radioligand [^{125}I]ICYP in a final volume of 500 μl. The non-specific binding is evaluated using 10 μM of alprenolol.

Under these conditions, the β2AR activity is detected in the cell culture supernatants from 48 hours after the infection, reaches its maximum 72 hours after the infection, and remains constant up to 120 hours after the infection.

These results are illustrated in FIG. 1.

This figure also shows the results obtained, under the same experimental conditions, on culture supernatants of cells infected both with the recombinant baculovirus c-myc-β2AR, and a recombinant baculovirus expressing the HIV Gag protein (●=β2AR; ♦=β2AR+Gag). It is observed that, contrary to what was initially expected, the presence of the Gag protein does not increase the quantity of β2AR in the culture supernatants.

It is also observed that the β2AR activity detected in the supernatants does not come from the cell lysis, since this activity appears 48 hours after the infection; that is to say at a time where the majority of the infected cells are still viable, and does not increase between 72 and 120 hours after the infection, in spite of the substantial cell lysis which occurs at this time.

The nature of the particles in the supernatant carrying the β2AR activity was checked by electron microscopy after labelling these particles with the aid of an antibody directed against the c-myc antigen, or an antibody directed against the β2AR receptor. It was thus observed that the particles recognized by either of these antibodies are 15×100 nm rods, which corresponds to extracellular baculoviruses.

In the case of the co-infection with a baculovirus expressing the Gag protein, the presence of particles exhibiting the morphology of the Gag particles, and which are recognized by an anti-Gag antibody, is observed, in addition, in the supernatant; however, unlike the extracellular baculoviruses, these Gag particles are only very weakly recognized by the anti-c-myc and antiβ2AR antibodies.

The presence of the β2AR receptor was also verified in the preparations of recombinant baculovirus c-myc-β2AR which are purified on a sucrose gradient, as described in Example 1 b) above.

The β2AR activity was determined according to the protocol described in Example 2 above, on various fractions of the gradient.

In parallel, the detection of the vp80, gp67 and vp39 antigens of the baculovirus AcMNPV, using a polyclonal antibody directed against these antigens, was carried out on the same fractions. The results obtained show that the β2AR activity cosediments with the viral particles.

All the results obtained above show that not only are molecules of the receptor expressed in the recombinant extracellular baculoviruses, but also that they are active molecules.

The quantification of the β2AR activity in the recombinant extracellular baculovirus preparations purified on a sucrose gradient makes it possible to evaluate the density of the active receptor at about 25 pmol/mg of total proteins.

EXAMPLE 3

Comparison of the Forms of the β2AR Receptor Which are Present in Cell Membrane Preparations and in the Extracellular Baculoviruses

Sf9 cells infected with the recombinant baculovirus c-myc-β2AR are harvested 72 hours after the infection. The extracellular baculoviruses c-myc-β2AR are harvested from the culture supernatant of these cells, and the viral particles are purified as described in Example 1b).

The membranes of the Sf9 cells are prepared as follows: the cells are centrifuged at 500×g for 5 minutes at 4° C., rinsed once with PBS buffer at 4° C., and resuspended in lysis buffer (20 mM Tris-HCl, 5 mM EDTA, pH 7.4 containing 5 μg/ml leupeptin, 5 μg/ml of trypsin inhibitor and 10 μg/ml of benzamidine) at 4° C. The cells are then lysed by sonication, the lysates are centrifuged for 5 min at 500×g at 4° C. and the supernatants centrifuged at 45,000×g for 20 min at 4° C. The pellets are resuspended at 4° C. in reaction buffer (75 mM Tris-HCl (pH 7.4), 12.5 mM magnesium chloride, 2 mM EDTA), in the presence of protease inhibitors.

6 mg of the cell membrane preparation or of the purified baculovirus preparation are added to 5 ml of solubilization buffer (10 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4, 0.3% n-dodecyl maltoside (BOEHRINGER MANNHEIM) in the presence of protease inhibitors. The solubilization is carried out for 90 min at 4° C.

The solubilized receptors are purified by affinity chromatography as described below. The ALPRENOLOL-SEPHAROSE affinity matrix is synthesized according to the method of BENOVIC et al. [J. Biol. Chem., 262 :9026–9032, (1987)]. This matrix is used to purify c-myc-β2AR according to the protocol described by MOUILLAC et al. [J. Biol. Chem., 267 :21733–21737, (1992)]. All the buffers comprise n-dodecyl maltoside (0.05%).

The preparations obtained after affinity chromatography are concentrated using CENTRIPREP and CENTRICON cartridges (AMICON)and the quantity of c-myc-β2AR in each sample is determined using [^{125}I]-iodocyanopindolol ([^{125}I]ICYP) as described by MOUILLAC et al. [J. Biol. Chem., 267 :21733–21737, (1992)]. The preparations of viral particles, of membranes or of β2AR purified by affinity chromatography are subjected to a polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), under non-reducing conditions, on 10% gel plates. The proteins separated on the gels are transferred onto nitrocellulose and revealed with an anti-c-myc mouse monoclonal antibody, and a second anti-mouse antibody coupled to alkaline phosphatase or to horseradish peroxidase. The results are illustrated by FIG. 2.

The Western blotting of the cell membrane preparation (FIG. 2, lane 1) shows the presence of several immunoreactive bands, between 40 and 50 kDa.

The Western blotting of the preparations of β2AR purified by affinity chromatography (FIG. 2, lane 2) shows a single and broad immunoreactive band, between 46 and 50 kDa, which represents the biologically active mature form of the β2AR receptor.

The Western blotting of the purified extracellular baculovirus preparation (FIG. 2, lane 3) also shows the presence of a single and broad immunoreactive band between 46 and 50 kDa.

These results show that the β2AR receptor molecules present in the extracellular baculoviruses represent only the

biologically active form, unlike the β2AR receptor molecules present in the cell membrane preparations, which represent a mixture of active and inactive forms.

EXAMPLE 4

Pharmacological Properties of Various Receptors Expressed in the Extracellular Baculoviruses

Preparations of extracellular baculoviruses expressing the β2AR, M1 or D1 receptors are obtained as described in Example 1 above.

The binding of each of the receptors to the ligand is evaluated as described in Example 2 above.

The competitive binding trials in the presence of agonists are carried out using 70 pM of [^{125}I]ICYP as radioligand. The concentration of the non-labelled ligand varies from 10^{-4} to 10^{-12} M.

The saturation assays of the M1-muscarinic (M1-R) and D1-dopaminergic (D1-R) receptors expressed in the viral particles are carried out using 1–100 nM [3H]pirenzepine (NEN, DUPONT) and 0.02–3 nM [^{125}I]-R(+)SCH-23390 (NEN, DUPONT) with 5–10 μg or 1–2 μg of protein for M1-R and D1-R respectively. To evaluate non-specific binding, 1 μM atropine (RBI) is added to the reaction mixture for M1-R, and 10 μM haloperidol (RBI) for D1-R.

The results of these experiments are illustrated by Table I below.

TABLE I

Receptor	Ligand	Kd pM	BMax pmol/mg of protein	Ki μM
β2AR	[^{125}I] ICYP	49.4 ± 11.5		
	Epinephrine			8.98 ± 4.02
	Arterenol			3.27 ± 0.38
M1	[^{3}H] -Pirenzepine	1360 ± 670	5.56 ± 0.46	
D1	[^{125}I] -SCH23390	118 ± 63	5.21 ± 0.84	

These results show that various receptors of the G-protein-coupled receptor family are expressed in an active form in extracellular baculoviruses.

EXAMPLE 5

Palmitoylation of the β2AR Receptor Expressed in Extracellular Baculoviruses

The viral particles expressing c-myc-β2AR are prepared as described in Example 1 above, and the pellet resuspended in PBS. 1 mCi of [^{3}H]palmitate dissolved in dimethyl sulphoxide is added to the viral particles. The reaction is carried out for defined periods in the presence or the absence of 1 μM (final concentration) of isoproterenol.

The results are illustrated by FIG. 3:

Legend to FIG. 3:

□: incorporation in the absence of isoproterenol;

□: incorporation in the presence of isoproterenol.

EXAMPLE 6

Comparison of the Forms of the β2AR Receptor

The viral particles expressing c-myc-β2AR are prepared as described in Example 1 above, and the pellet resuspended in a buffer (100 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4 and protease inhibitors). 1 volume of extracellular baculoviruses and 1 volume of phosphorylation mixture (2.3 μCi/pl of [γ^{32}P]ATP, 10 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.4, 25 mM phosphoenol pyruvate, 0.3 mM GTP, 1 mM ATP, 4 U/ml of pyruvate kinase and 20 U/ml of myokinase) are mixed. The reaction is carried out for 25 min at 30° C. At the end of the reaction, the incorporation of ^{32}P is measured in the absence of activator (control) or in the presence of 1 μM of isoproterenol, or of 100 μM of dibutyril cyclic AMP, or of 100 μM of forskolin. The results are illustrated by FIG. 4.

Legend to FIG. 4:

On the y-axis: relative incorporation of ^{32}P (arbitrary units)

On the x-axis:

BASAL: control

FRSK: incorporation in the presence of forskolin

cAMP: incorporation in the presence of dibutyril cyclic AMP

ISO: incorporation in the presence of isoproterenol

EXAMPLE 7

Functionality of the β2AR Receptor Expressed in Extracellular Baculoviruses

The viral particles expressing c-myc-β2AR are prepared as described in Example 1 above, and the pellet resuspended in a buffer (75 mM Tris-HCl, 12.5 mM $MgCl_2$, 2 mM EDTA, pH 7.4 and protease inhibitors). 20 μl of extracellular baculovirus suspension are mixed with 30 μl of reaction medium containing 0.2 mM ATP, 0.090 mM GTP, 0.20 mM cAMP, 0.20 mM isobutylmethylxanthine, 1 μCi [γ^{32}P]ATP, 5 mM phosphoenol pyruvate, 0.3 U of pyruvate kinase and 2 U of myokinase. After incubating for 30 min at 37° C., the reactions are stopped by the addition of 1 ml of stop solution (0.4 mM ATP, 0.3 mM cyclic AMP and 25,000 cpm of tritiated cyclic AMP). The activity was determined in the absence of activator (control) or in the presence of one of the following activators: 1 μM of isoproterenol, 10 μM NaF or 100 μM of forskolin. The results are expressed in picomoles of cyclic AMP produced per minute and per milligram of protein. These results are illustrated by FIG. 5.

Legent to FIG. 5:

On the y-axis: adenyl cyclase activity (in picomoles of cyclic AMP/min/mg of protein)

On the x-axis:

BASAL: control

FRSK: incorporation in the presence of forskolin

NaF: incorporation in the presence of NaF

ISO: incorporation in the presence of isoproterenol

These results show that the β2AR receptor present in the extracellular baculoviruses is in an environment which reproduces the natural membrane environment, and that the extracellular baculovirus preparations can therefore be used in all membrane receptor applications where a reproduction of this environment is desirable.

What is claimed is:

1. Isolated baculovirus viral particles obtained by a method of producing a recombinant membrane receptor protein comprising:

introducing a baculovirus expression vector encoding said membrane receptor protein into an insect host cell;

culturing the resultant insect host cell for a sufficient time to permit expression of said membrane receptor protein and baculovirus viral particles;

separating the cells from the baculovirus viral particles; and

isolating the baculovirus viral particles.

2. The recombinant baculovirus viral particles of claim 1, wherein said membrane receptor protein belongs to the superfamily of receptors having seven transmembrane domains.

3. The recombinant baculovirus viral particles of claim 2, wherein said membrane receptor protein belongs to the family of G-protein-coupled receptors.

4. The recombinant baculovirus viral particles of claim 4, wherein at least 90% of said membrane receptor protein is in active form.

5. The recombinant baculovirus viral particles of claim 1, wherein at least 95% of said membrane receptor protein is in active form.

* * * * *

EXHIBIT C

US007070978B2

(12) **United States Patent**
Hamakubo et al.

(10) **Patent No.: US 7,070,978 B2**
(45) **Date of Patent: Jul. 4, 2006**

(54) **METHOD FOR EXPRESSING AND PURIFYING PROTEINS USING BUDDED BACULOVIRUS**

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(73) Assignee: **Toudai TLO, Ltd.**, Tokyo (JP)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 33 days.

(21) Appl. No.: **09/884,481**

(22) Filed: **Jun. 20, 2001**

(65) **Prior Publication Data**

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(51) **Int. Cl.**

C12N 7/02	(2006.01)
C12N 15/86	(2006.01)
C12N 15/09	(2006.01)

(52) **U.S. Cl.** **435/239**; 435/456; 435/69.7

(58) **Field of Classification Search** 435/320.1, 435/325, 7.2, 41, 69.1, 69.2, 70.1, 70.2, 5, 435/71.1, 235.1, 239; 530/344

See application file for complete search history.

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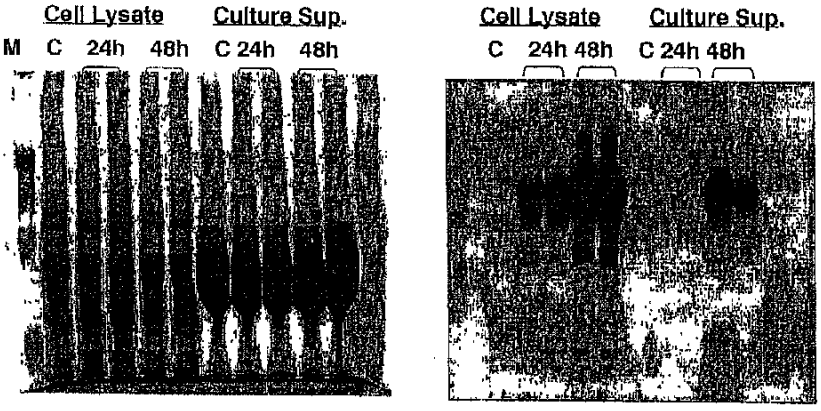
Assistant Examiner—M. Franco Salvoza
(74) *Attorney, Agent, or Firm*—Greenblum & Bernstein P.L.C.

(57) **ABSTRACT**

Method for efficiently expressing a protein in an active form by using baculovirus expression system wherein the protein is selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein. There is provided a method for expressing such a protein by culturing a host infected with at least one type of recombinant baculovirus which contains a gene encoding the protein, wherein the protein is expressed in a budded baculovirus released from the host.

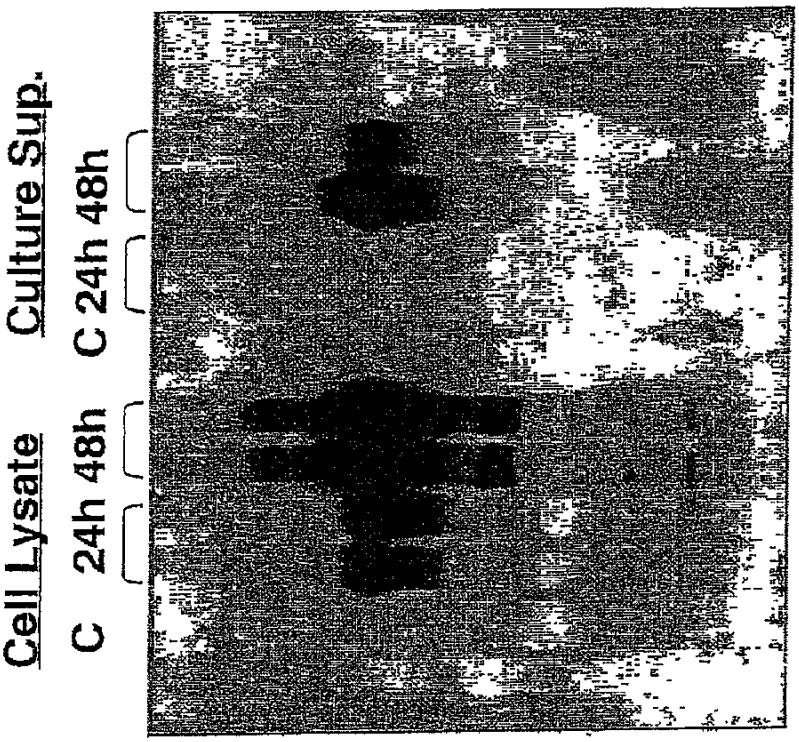
22 Claims, 9 Drawing Sheets

Expression of SREBP2 in Sf9 cells



CBB staining

Immunoblot staining with anti-SREBP2



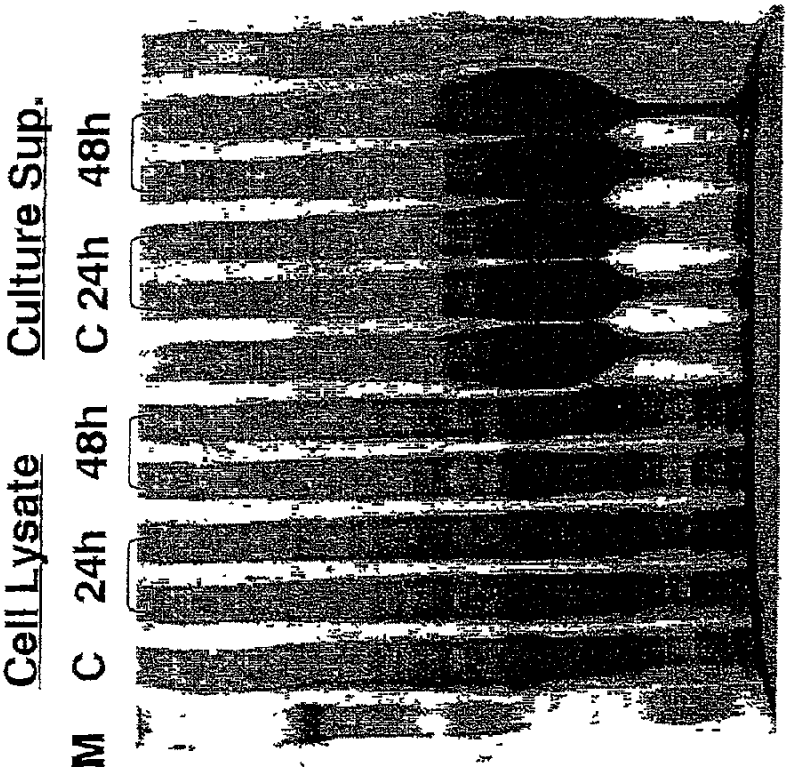
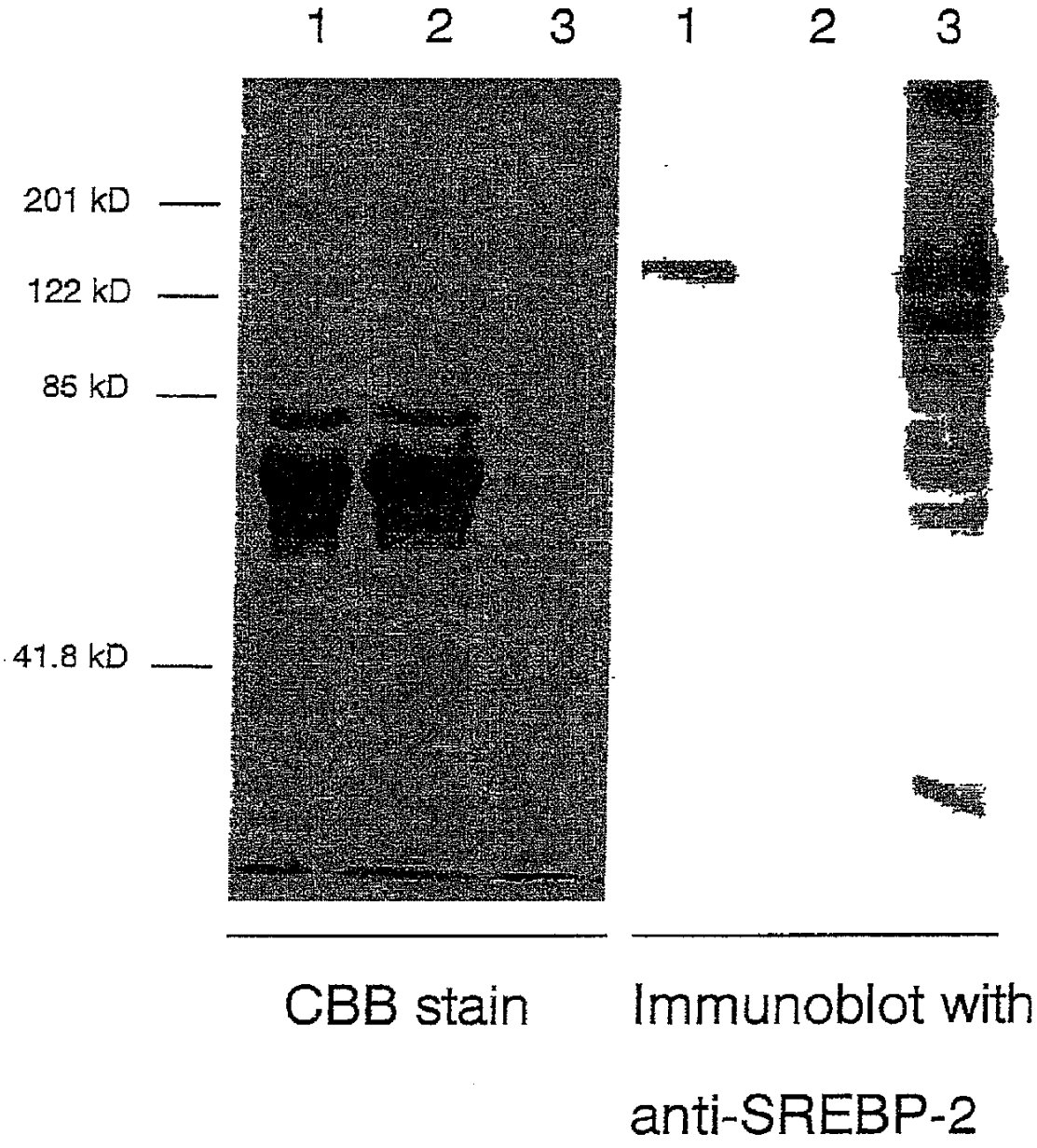


Fig. 1 Expression of SREBP2 in Sf9 cells

Fig.2 Centrifugation separation of SREBP2 expressed in culture supernatant



Lane 1 800g 10min Supernatant
Lane 2 40000g 20min Supernatant
Lane 3 40000g 20min pellet

Fig.3 Density gradient centrifugation of 40000g pellet

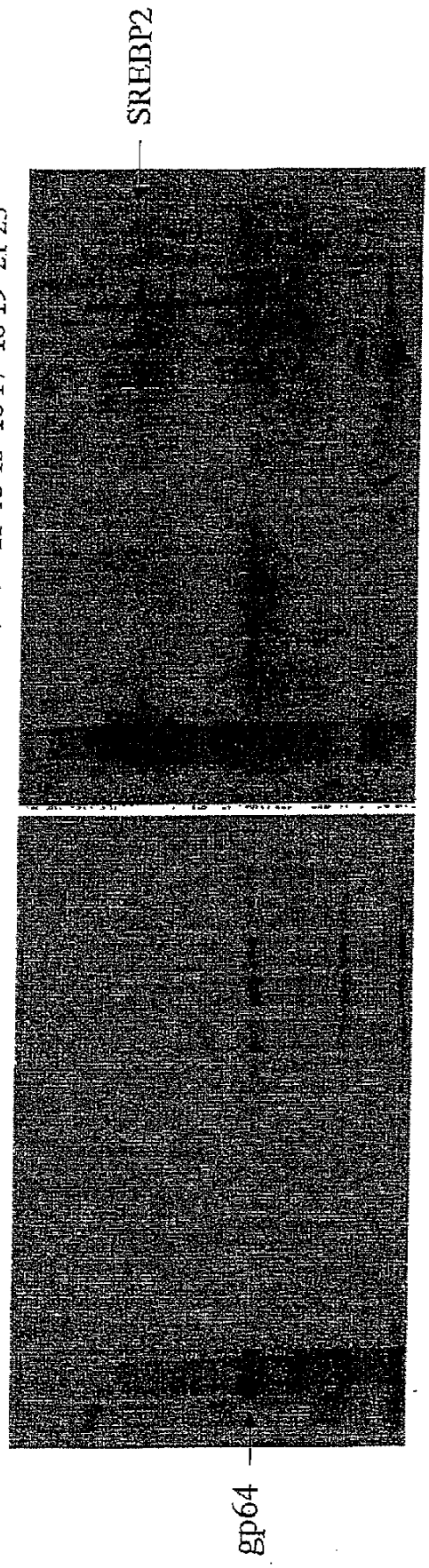
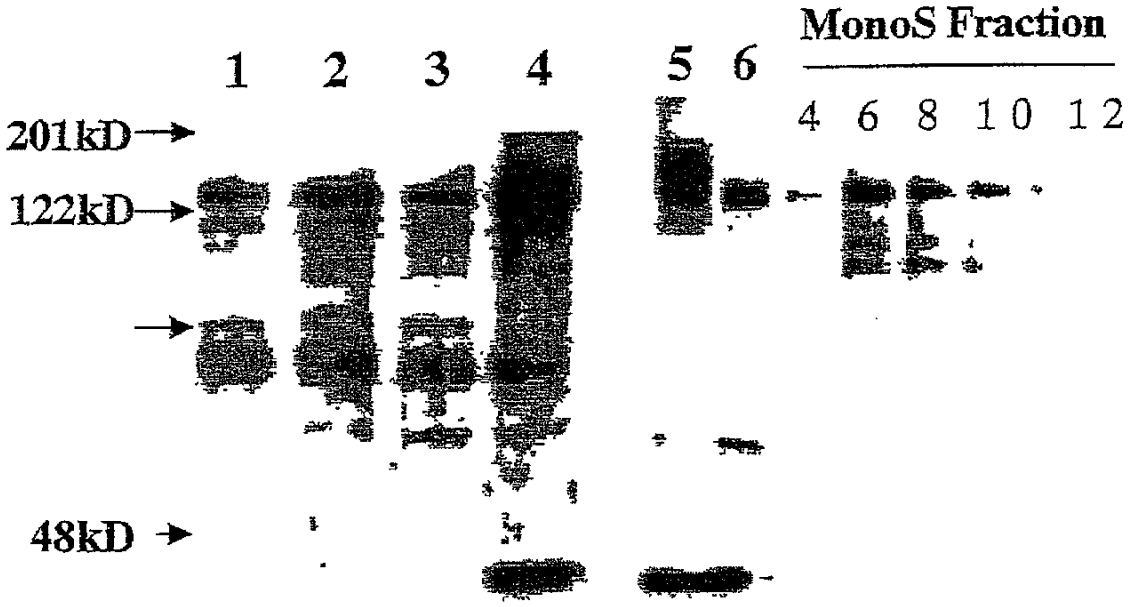


Fig.4 Solubilization and Purification of SREBP from Budded virus



Immunoblot with anti-SREBP
Lane 1: Solubilized SREBP
Lane 2, 3: Affinity column pass through
Lane 4, 5: 10M urea eluate from affinity column
Lane 6: PD10 gel filtration

Fig.5 Preparation of mouse anti - serum

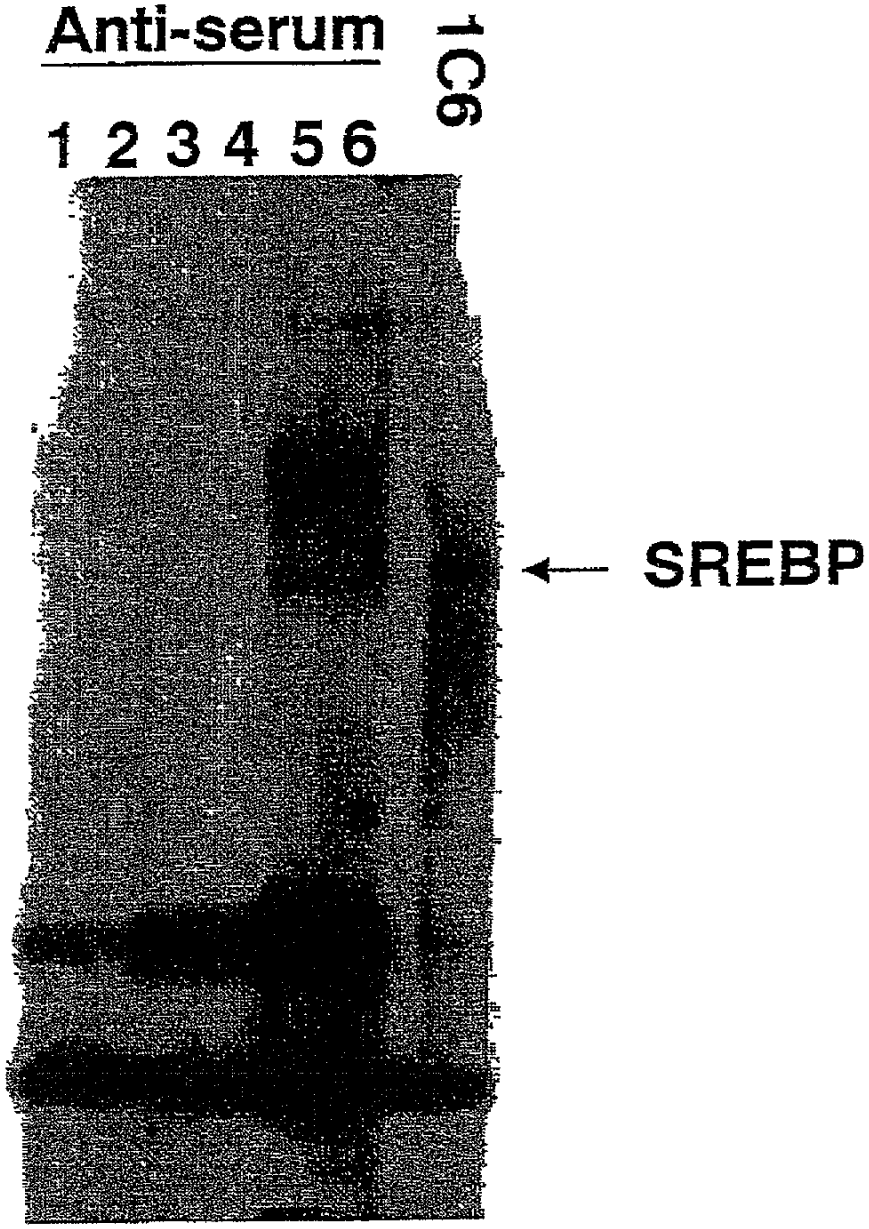
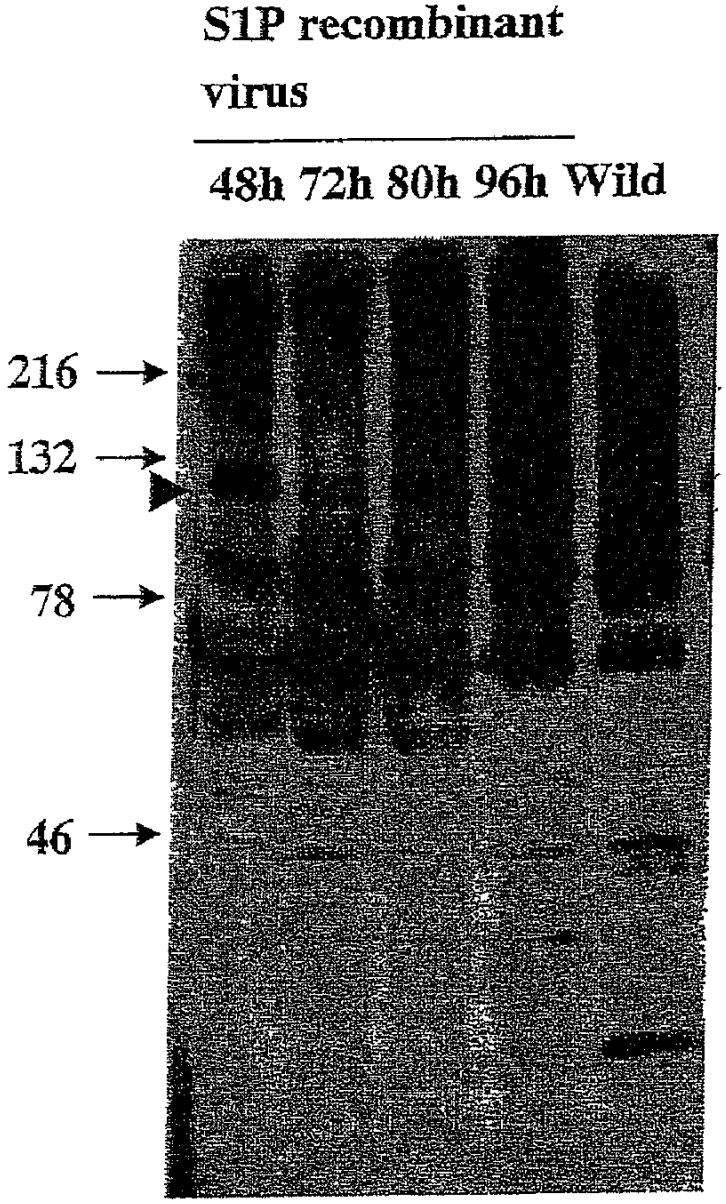


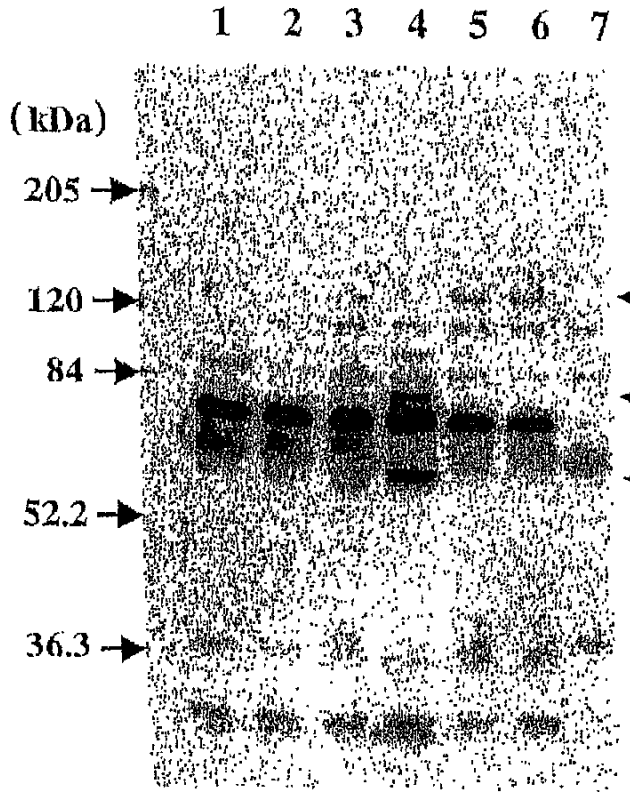
Fig.6 Expression of S1P in Budded virus



Immunoblot with anti-S1P serum.
Arrow head; S1P

Fig.7 **Cleavage of SREBP on BV by S1P on BV**

Immunoblot analysis with R004 (anti- N-terminal Domain of SREBP)

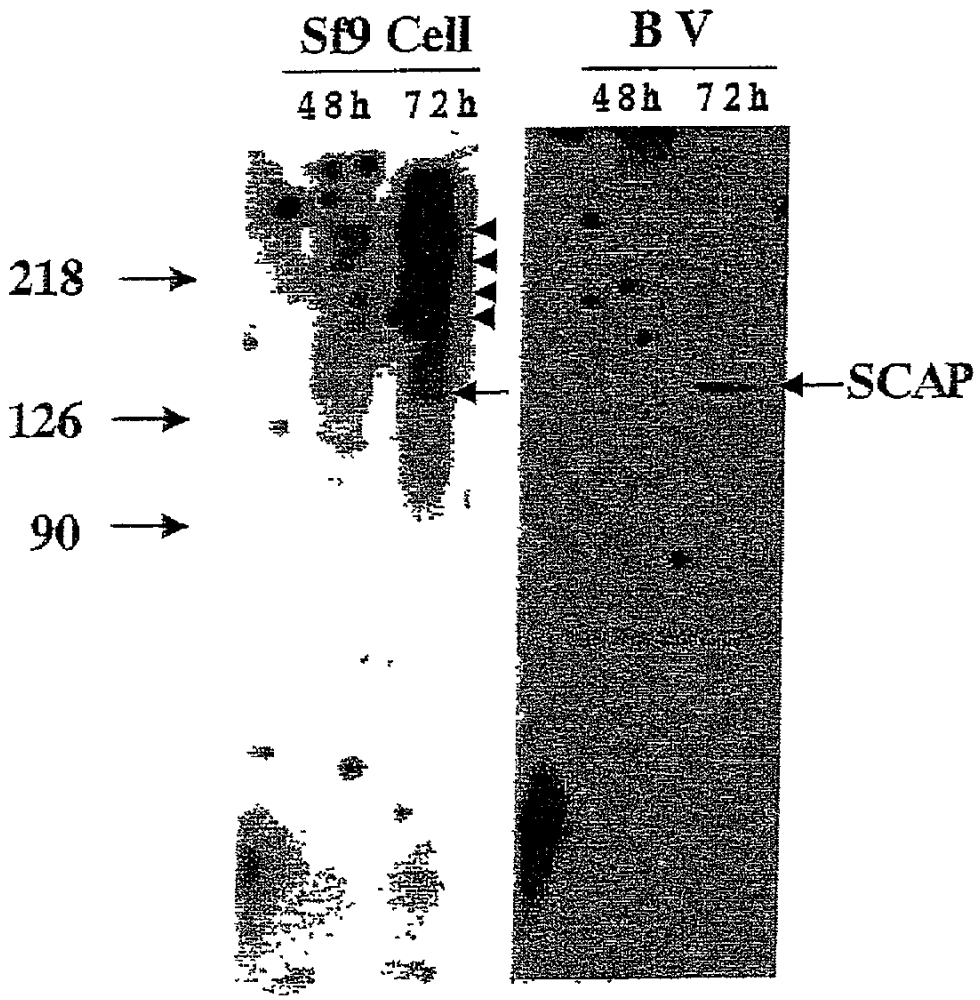


8% SDS-PAGE

lane 1 : SREBP (preincubation)
2 : SREBP
3 : SREBP + S1P
4 : SREBP + S1P + 5 mM Ca
5 : SREBP + S1P + 10 mM EGTA
6 : SREBP + S1P + 10 mM EDTA
7 : S1P

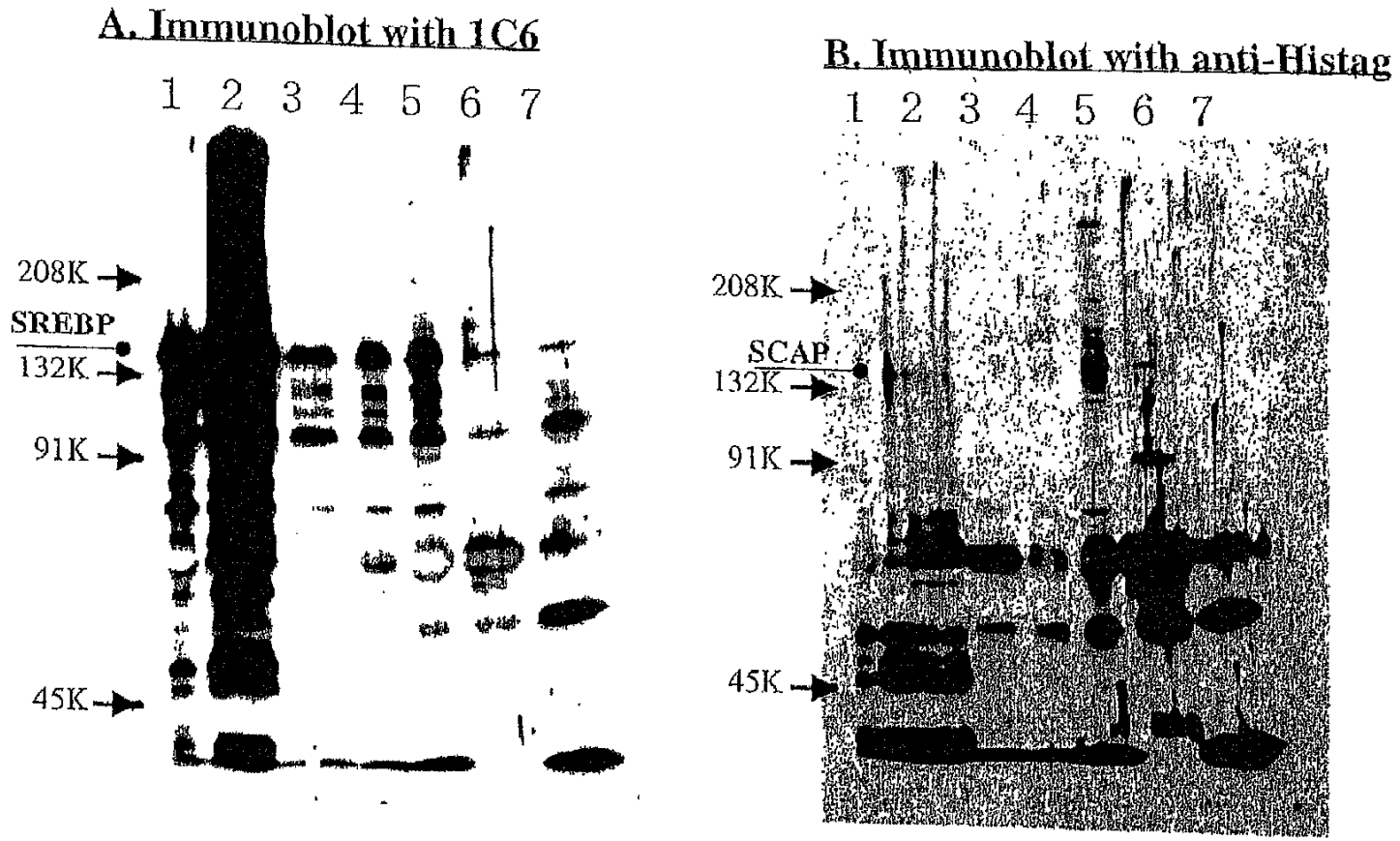
lane 2 - 7: Samples were incubated at 37℃ for 12 hr.

Fig.8 Expression of SCAP in budded virus



Immunoblot with anti-His Tag

Fig.9 Coexpression of SREBP2 and SCAP



Lane1. Budded virus 2. Solubilized pellet 3.Solubilized fraction 4. Ni-NTA immunoprecipitation supernatant
5. 1C6 immunoprecipitaion supernatant 6. Ni-NTA immunoprecipitation pellet
7. 1C6 immunoprecipitation pellet

METHOD FOR EXPRESSING AND PURIFYING PROTEINS USING BUDDED BACULOVIRUS

TECHNICAL FIELD TO WHICH THE INVENTION BELONGS

The present invention relates to a technology for expressing a protein present on a membrane such as an endoplasmic reticulum membrane or a Golgi apparatus membrane using a baculovirus expression system. More particularly, the present invention relates to a method for expressing a membrane protein in a budded baculovirus by culturing a host infected with at least one type of recombinant baculovirus which contains a gene encoding the membrane protein.

BACKGROUND OF THE INVENTION

A baculovirus expression system enables high-level expression of a gene of interest by causing recombination of the gene in Sf9 cells using a promoter of a polyhedrin gene of baculovirus. A polyhedrin is expressed in the nucleus of Sf9 cell at a high level as an occlusion body which is a form to be used when viruses become latent within the cell. The baculovirus expression system where a recombinant protein is introduced into a polyhedrin gene and the expressed proteins are purified, has many advantages over that of *Escherichia coli*, such that the expressed proteins are hard to agglutinate and the expressed proteins undergo a posttranscriptional modification which is necessary for protein functions, such as addition of sugar chains and coordination of metal ion.

Baculovirus has another life cycle. In order to proliferate and infect, baculovirus becomes a budded virus (Budded virus: this is also referred to as budded baculovirus in this specification), rupturing Sf9 cell membrane and being released outside the cell. Bouvier et al have reported that at this time a receptor of seven-transmembrane type recombined into the above polyhedrin protein is expressed on the cell membrane and recovered from the envelope of the budded baculovirus (Loisel T P, Ansanay H, St-Onge S, Gay B, Boulanger P, Strosberg A D, Marullo S, Bouvier M., Nat Biotechnol. 1997 November; 15(12): 1300–4., Recovery of homogeneous and functional beta 2-adrenergic receptors from extracellular baculovirus particles; and International Publication WO98/46777). It has also been reported that, whereas most receptors of seven-transmembrane type expressed in a host cell have a sugar chain structure which is not functional, only functional receptors are recovered from the viral envelope. However, Bouvier et al have not mentioned membrane proteins other than receptor proteins.

SUMMARY OF THE INVENTION

A problem to be solved by the present invention is to provide a method for efficiently expressing a protein in an active form by using baculovirus expression system wherein the protein is selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein. Another problem to be solved by the present invention is to provide a method for producing antibodies against the above expressed proteins by using the above method, and a method for screening a chemical substance useful as drugs and the like by using the above method.

SREBP (sterol regulatory element binding protein) 2, HMG-CoA (hydroxymethylglutaryl coenzyme A) reductase, SCAP (SREBP cleavage activating protein), and S1P (site 1 protease) are of a membrane protein group which is distributed over the endoplasmic reticulum (ER) membrane or the Golgi apparatus membrane, and is involved in intracellular cholesterol feedback regulation. The present inventors have succeeded in recovering these proteins from extracellular budded viral envelopes by allowing Sf9 cells to express these proteins using a baculovirus expression system. It was also found that an ER membrane protein recovered from the viral envelope forms a single band and has a higher stability as compared to a membrane protein recovered from Sf9 cell membrane with many degradation products. The present invention have been completed based on these findings.

According to the present invention, there is provided a method for expressing a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein by culturing a host infected with at least one type of recombinant baculovirus which contains a gene encoding said protein, wherein said protein is expressed in a budded baculovirus released from said host.

According another aspect of the present invention, there is provided a method for preparing a protein which comprises:

culturing a host infected with a recombinant baculovirus which contains a gene encoding a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein;

recovering a budded baculovirus released from said host; and

recovering the protein expressed from said budded baculovirus.

Preferably, the protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein is a membrane-bound protein of a cell organelle.

Preferably, the protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein is SREBP2, HMG-CoA reductase, S1P, or SREBP cleavage activating protein.

Preferably, the host is an insect cell or an insect larva.

According to further another aspect of the present invention, there is provided a budded baculovirus which is released from a host infected with at least one type of baculovirus which contains a gene encoding a protein

selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein.

According to still further another aspect of the present invention, there is provided a method for screening a chemical substance which comprises measuring interaction between said protein and other chemical substances using the budded baculovirus of the present invention.

Preferably in the present invention, a drug which inhibits or activates a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein, is screened.

According to still further another aspect of the present invention, there is provided a method for screening a chemical substance which activates or inhibits the function of two or more proteins selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein, by co-infecting two or more different types of recombinant baculoviruses, each of which contains a gene encoding a different type of said protein; and co-expressing the function of said two or more proteins.

According to still further another aspect of the present invention, there is provided a method for producing an antibody against a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein, wherein the budded baculovirus of the present invention is used as an immunogen, as well as an antibody which is produced by this method.

In one aspect, the present invention is directed to a method for recovering a budded baculovirus expressing an intracellular organelle membrane-bound protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein comprising culturing a host infected with at least one recombinant baculovirus which contains a gene encoding said protein, expressing said protein in said infected host allowing baculovirus produced in said host to bud and be released from said host with said expressed protein being in the envelope of said budded baculovirus, and separating the budded baculovirus.

In another aspect, the present invention is directed to a method for preparing an intracellular organelle membrane-bound protein which comprises culturing a host infected with a recombinant baculovirus which contains a gene encoding a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein; recovering a budded baculovirus released from said host; and recovering the protein expressed in said infected host allowing baculovirus produced in said host to bud and be released from said host with said expressed protein being in the envelope of said budded baculovirus.

In still another aspect, the present invention is also directed to a method for recovering a budded baculovirus expressing a non-receptor protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein comprising culturing a host infected with at least one recombinant baculovirus which contains a gene encoding said protein, expressing said protein in said infected host allowing baculovirus produced in said host to bud and be released from said host with said expressed protein being in the envelope of said budded baculovirus, and separating the budded baculovirus.

In still another aspect, the present invention is directed to a method for preparing a non-receptor protein which comprises culturing a host infected with a recombinant baculovirus which contains a gene encoding a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein; recovering a budded baculovirus released from said host; and recovering the protein expressed in said infected host allowing baculovirus produced in said host to bud and be released from said host with said expressed protein being in the envelope of said budded baculovirus.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** is an illustration showing the expression of SREBP2 in Sf9 cells.

FIG. **2** is an illustration showing the result of centrifugation of SREBP2 expressed in the culture supernatant.

FIG. **3** is an illustration showing the result of density-gradient centrifugation of 40,000 g pellet.

FIG. **4** is an illustration showing the results of solubilization and purification of SREBP from the budded virus.

FIG. **5** is an illustration showing the result of production of a mouse anti-serum against SREBP.

FIG. **6** is an illustration showing the result of expression of S1P in the budded virus.

FIG. **7** is an illustration showing cleavage of SREBP on the budded virus by S1P on the budded virus.

FIG. **8** is an illustration showing the result of expression of SCAP in the budded virus.

FIG. **9** is an illustration showing the result of co-expression of SREBP2 and SCAP in the budded virus.

DETAILED DESCRIPTION OF THE INVENTION

The embodiments and methods of the present invention will be described below in detail.

The method of the present invention relates to a method for expressing a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein by culturing a host infected with at least one type of recombinant baculovirus which contains a gene encoding the protein, wherein the protein is expressed in budded baculovirus which is released from the host.

The term "membrane-bound" as used herein broadly means that a protein exists in cell membrane or in the membrane of intracellular organelle (e.g. endoplasmic reticulum or Golgi apparatus), and the type of the proteins is not specifically limited. Preferably, a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein, is a membrane-bound proteins of intracellular organelles such as proteins bound to the membranes of endoplasmic reticulum and of Golgi apparatus.

Membrane-bound enzymes include, for example, HMG-CoA reductase involved in cholesterol metabolism, ACAT (acyl-coenzyme A: cholesterol acyltransferase), and 7. -hydroxylase. Other examples are the cytochrome P450 system involved in detoxification, ATP synthase present in mitochondria, cytochrome oxidase and reductase, and electron transport system enzymes such as NADH-Q reductase. Still other examples include: a processing protease family involved in processing of hormones, controlling factors, nutritional factors and the like, such as S1P (site 1 protease), furin, PC (proprotein convertase), S2P (site 2 protease), endothelin converting enzyme, angiotensin converting enzyme, and neprilysin; an ADAMS (a disintegrin and metalloprotease) family which is involved in signal transduction system such as notch signal; β-selectase and γ-selectase which cleave amyloid precursor proteins (e.g. presenillin); and a matrix metalloprotease family which is involved in the decomposition of the extracellular matrix. Other examples are membrane lipid metabolizing enzymes such as diacylglycerol synthase, phosphatidic acid phosphatase, and phosphatidylserine synthase; and enzymes involved in signal transduction such as adenylate cyclase.

Examples of membrane-bound enzyme-substrate proteins include those involved in signal transduction and transcriptional control, such as sterol regulatory proteins (SREBP), Notch, Ire1, and ATF6, and other amyloid precursor proteins, TNF. (tumor necrosis factor) precursor, Stem cell factor, M-CSF (monocyte colony stimulating factor) precursor, and Klotho.

Examples of membrane-bound enzyme activators include presenillin, and SCAP (SREBP cleavage activating protein).

Examples of membrane-bound transport proteins include NPC (Niemann-Pick type c) 1 which transport lipid such as cholesterol, ABC (ATP-binding cassette) transporter, caveolin and fatty acid transporter; as well as sugar transporters including glucose transporters such as GLUT1-4 and amino acid transporters such as glutamate transporter and serotonin transporters. Examples of membrane proteins involved in a transportation of a substance between intracellular vesicles include Sec12.

Other examples are channel proteins which allow selective passage of membrane impermeable molecules under certain condition. Such channel proteins include aquaporin family which is selective channels for water, and ion channels which are selective channels for potassium ions, calcium ions, sodium ions and the like.

Examples of membrane structural proteins and proteins involved in adhesion include NCAM (Neural cell adhesion molecule), ICAM (intercellular adhesion molecule), the cadherin family, integrin, desmocholine, desmograin, L-selectin, connexin, and glycoproteins. Other examples include major histocompatibility complex (MHC) involved in antigen presentation in immunocytes, and chaperone proteins such as calnexin, PDI (protein disulfide isomerase), CFTR (cystic fibrosis transmembrane conductance regulator), and major prion protein precursor (prion), which are thought to be involved in the formation of high dimensional structure of a protein.

From the above, it is apparent that the proteins according to the present invention include intracellular organelle proteins and/or non-receptor proteins.

In the present invention, at least one type of recombinant baculovirus containing a gene encoding the above-mentioned protein to be expressed is employed.

Baculoviruses which infect insects and cause diseases are envelope viruses having cyclic double stranded DNAs as gene, and shows a sensitivity to insects of the orders Lepidoptera, Hymenoptera, Diptera and the like. Among the baculoviruses, nuclear polyhedrosis virus (NPV) refers to a group which produces a large amount of occlusion bodies called polyhedra within the nucleus of an infected cell. A polyhedron comprises polyhedrin proteins having a molecular weight of 31 kDa, and is produced in a large amount at late stage of the infection and have many viral particles embedded therein. Since polyhedra is essential for the viruses to survive in nature, but unnecessary for the proliferation of a virus, the viruses can infect and proliferate even if a foreign gene to be expressed is inserted in place of a polyhedron gene.

As baculoviruses used in the present invention, a virus vector such as *Autographa californica* NPV (AcNPV) of the subfamily Phytometra and *Bombyx mori* NPV (BmNPV) of silkworm, can be used.

Example of a host of AcNPV (infected and established cells) include *Spodoptera frugiperda* cell (Sf cell), and that of BmNPV (infected and established cells) include BmN4 cell. Vectors of AcNPV line are preferred because Sf cell possesses a higher proliferation rate compared to BmN4 cells and the like, and AcNPV possesses infectivity to human liver cells, human fetal nephrocytes and the like.

As hosts, for example, *Spodoptera Frugiperda* cell lines Sf9 and Sf21 have been established from ovarian tissue of *S. frugiperda* larva and are available from Invitrogen, Pharmingen (San Diego, Calif.), ATCC or the like. In addition, a living insect larva can also be used as a host.

A method for constructing a recombinant virus used in the present invention may be performed by standard techniques, and it can be performed by, for example, the following steps.

First, a recombinant transfer vector is constructed by inserting a gene of a protein to be expressed into the transfer vector.

The whole size of the transfer vector generally ranges from about several kb to about 10 kb where about 3 kb of the

size corresponds to a backbone derived from a plasmid, which contains an antibiotic resistance gene (e.g. resistance to ampicillin) and a signal to initiate DNA replication in bacteria. In addition to this backbone, a transfer vector generally contains several kb each of the 5' and 3' regions of a polyhedron gene, so that, when transfection as described below is performed, homologous recombination between the gene of interest and the polyhedron gene occurs between these sequences. Preferably, the transfer vector contains a promoter for the expression of a gene of a protein. Examples of promoters include a polyhedron gene promoter, a p10 gene promoter, and a capsid gene promoter.

Types of transfer vectors are not specifically limited. Examples of AcNPV line transfer vectors include pEVmXIV2, pAcSG1, pVL1392/1393, pAcMP2/3, pAcJP1, pAcUW21, pAcDZ1, pBlueBacIII, pAcUW51, pAcAB3, pAc360, pBlueBacHis, pVT-Bac33, pAcUW1, and pAcUW42/43; those of BmNPV line transfer vectors include pBK283, pBK5, pBB30, pBE1, pBE2, pBK3, pBK52, pBKblue, pBKblue2, pBF series (all of which are available from FUNAKOSHI, Fujisawa Pharmaceutical Co., Ltd. and the like).

Next, in order to prepare recombinant viruses, the above-mentioned recombinant transfer vectors are mixed with viruses, and then transferred into cultured cells to be used as a host, or alternatively are transferred into cultured cells to be used as host which have been previously infected with viruses, so as to cause homologous recombination between the recombinant transfer vectors and viral genome DNAs, thereby constructing recombinant viruses.

Cultured cells used as a host are the above-mentioned host, and generally include insect culture cells (e.g. Sf9 cells, BmN cells, etc.). Culturing conditions are determined appropriately by persons skilled in the art. When Sf9 cells are used, culturing is preferably performed at around 28° C. in a medium containing 10% fetal calf serum. The thus constructed recombinant viruses can be purified by standard techniques such as plaque assay. The thus prepared recombinant viruses can be easily distinguished from non-recombinant viruses since they cannot form polyhedra because of the foreign DNA replaced or inserted in the region of the gene for the polyhedron protein of the nuclear polyhedrosis virus.

In the method of the present invention, the protein of interest can be recovered by allowing the above-mentioned recombinant baculovirus to infect the above-described appropriate host (culture cells of e.g. *Spodoptera Frugiperda* cell lines Sf9 and Sf21 or insect larvae) and recovering extracellular budded virus (BV) from the culture supernatant by separation means such as centrifugation after a certain period of time (e.g. 72 hours). Only one type of recombinant baculovirus may be infected, or two or more types of recombinant baculovirus may be co-infected.

Extracellular budded baculoviruses can be recovered, for example, as described below.

First, a culture solution of the infected cells is centrifuged at 500 to 1,000 g, thereby recovering the supernatant containing extracellular budded baculoviruses. The supernatant is centrifuged at about 30,000 to 50,000 g so as to obtain the precipitate containing extracellular budded baculoviruses. The precipitate is suspended in an appropriate buffer. The virus suspension is applied onto proper concentration gradients (e.g. sucrose sequential gradient), and then centrifuged at 100,000 g for fractionation. Finally, fractions containing desired proteins can be selected from the obtained fractions.

When the expressed proteins are obtained in the form of solubilized proteins, extracellular budded viruses are recovered by centrifugation at e.g. 40000 g from the culture solution of the infected cells. The recovered pellet is suspended in an appropriate buffer, treated with a dissolution agent such as lyso-phosphatidylcholine, and centrifuged at 30,000 rpm, thereby separating the suspension into a supernatant and a precipitate. The solubilized protein of interest is recovered in the supernatant.

The expressed protein recovered by the method of the present invention as described above is characterized in that it is recovered in its active form. Preferably, at least 50% or more, more preferably 60% or more, still more preferably 70% or more, further more preferably 80% or more, still more preferably 90% or more, and particularly preferably 95% or more of the protein is recovered in its active form by the method of the present invention. Such an active form of a membrane protein could not be recovered in a high yield by the conventional methods.

The present invention further provides a method for screening a chemical substance which comprises measuring interaction between other chemical substances and a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein, by using budded baculoviruses which are released from hosts infected with at least one type of recombinant baculovirus which contain a gene encoding said protein.

Examples of chemical substances to be screened include peptides, polypeptides, synthetic compounds, fermented products of microorganisms, extracts from organisms (including plant or animal tissues, microorganisms and cells) or libraries thereof. Examples of libraries include a synthetic compound library (e.g. a combinatorial library) and a peptide library (e.g. a combinatorial library). Chemical substances to be screened may be either natural or synthetic materials. Single candidate chemical substance may be individually tested, or a mixture of candidate chemical substances (including libraries) may be tested. Furthermore, a fractionated mixture such as a cell extract may be screened, followed by repeated fractionation, thereby isolating a substance with a desired activity.

These chemical substances are substances which are expected to interact with a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein. More preferably, these chemical substances are drugs which inhibit or activate the above-described proteins.

The present invention further provides a method for producing an antibody against a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein, which is characterized by using, as an immunogen, budded baculoviruses which are released by hosts infected with at least one type of recombinant baculoviruses that

contains a gene encoding a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein.

The preparation of antibodies can be performed by standard techniques. When a polyclonal antibody is produced, mammals are immunized as antigens with budded baculoviruses which are released from hosts infected with at least one type of recombinant baculovirus which contains a gene encoding a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein; blood is collected from the mammals; and antibodies are separated and purified from the collected blood. Examples of mammals which can be immunized include mice, hamsters, guinea pigs, chickens, rats, rabbits, dogs, goats, sheep, and cattle. Immunization can be performed by standard immunization techniques, for example by administering antigens once or more.

Preferably, antigens are administered twice or three times at an interval of 7 to 30 days, particularly 12 to 16 days, and the dose may be appropriately selected. The route of administration is not specifically limited and can be appropriately selected from subcutaneous administration, intracutaneous administration, intraperitoneal administration, intravenous administration, and intramuscular administration. Administration by intravenous, intraperitoneal, or subcutaneous injection is preferred. The antigen may be dissolved in an appropriate buffer such as that which contains a generally employed adjuvant, such as complete Freund's adjuvant, RAS [MPL(Monophosphoryl Lipid A)+TDM(Synthetic Trehalose Dicorynomycolate)+CWS(Cell Wall Skeleton) adjuvant system], or aluminum hydroxide. The above adjuvant may not be used depending on the administration route and the conditions.

The immunized mammals are raised for e.g. 0.5 to 4 months, and a small amount of the sera of the mammals is sampled from vena of the ears, and then the antibody titer is measured. When the antibody titer is increasing, antigen is administered at appropriate frequency depending on the condition. For example, booster immunization is performed using 100 μg to 1000 μg of antigen. One to 2 months after the final administration , blood is collected by standard techniques from the immunized mammals. The collected blood is separated and purified by standard methods such as precipitation using centrifugation, ammonium sulfate or polyethylene glycol, and chromatography including gel filtration chromatography, ion exchange chromatography and affinity chromatography, thereby obtaining desired polyclonal antibodies as polyclonal anti-sera.

When a monoclonal antibody is produced, a desired monoclonal antibody can be obtained by producing a hybridoma by cell fusion of an antibody-producing cell and a myeloma cell line. A hybridoma producing a monoclonal antibody can be obtained by a cell fusion method as described below.

Spleen cells, lymph node cells, B lymphocytes and the like from immunized animals are used as antibody-producing cells. Extracellular budded baculoviruses are used as antigens. Animals to be immunized include mice and rats. Antigens are administered to these animals by standard techniques. For example, a suspension or emulsion is prepared from an adjuvant such as a complete Freund's adjuvant and incomplete Freund's adjuvant and budded baculoviruses as antigens. The preparation is administered for example intravenously, subcutaneously, intracutaneously or intraperitoneally to animals several times, thereby immunizing the animals. Next, for example, spleen cells are obtained as antibody-producing cells from the immunized animals, and are fused with myeloma cells by a known method (G. Kohler et al., Nature, 256 495 (1975)), so that hybridomas can be prepared. Examples of myeloma cell lines used for cell fusion include mouse P3X63Ag8, P3U1 strain, and Sp2/0 strain. In the cell fusion, a fusion accelerator such as polyethylene glycol and Sendai viruses is used. For selection of hybridomas after cell fusion, a HAT (hypoxanthine aminopterin thymidine) medium can be used according to standard techniques.

Hybridomas obtained by cell fusion are cloned by limiting dilution and the like, and further screened, thereby obtaining a cell line which produces a monoclonal antibody specifically recognizing a desired protein.

To produce a monoclonal antibody of interest from the thus obtained hybridoma, the hybridoma is cultured by a standard cell culturing method or an ascite formation method, and then the monoclonal antibody can be purified from the culture supernatant or the ascite. Purification of monoclonal antibodies from the culture supernatant or the ascite can be performed by standard techniques. For example, an appropriate combination of ammonium sulfate fractionation, gel filtration, ion exchange chromatography, affinity chromatography and the like may be employed.

The present invention will be further explained by the following examples, but the invention is not limited by these examples.

EXAMPLES

Example 1

Purification of Sterol Regulatory Protein (SREBP 2) and Production of Antiserum

SREBP 2 is a transcriptional factor which is involved in transcriptional control of key enzymes, such as LDL receptor and HMG-CoA reductase, which are involved in intracellular control of cholesterol (Brown M S, Goldstein J., Proc. Natl. Acad. Sci. U.S.A., 1999 September 28; 96(20): 11041–8, A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood). A precursor protein of 125 kd is present as a protein of two-transmembrane type in the ER membrane. Cholesterol depletion within a cell causes SREBP 2 to separate from a transmembrane site by two steps of cleavage by protease and to be released into the cytoplasm. Then, SREBP 2 migrates to the nucleus and activates transcription by binding to a sre sequence on the promoter of a cholesterol controlling gene (Brown M S, Goldstein J., Proc. Natl. Acad. Sci. U.S.A. 1999 September 28; 96(20): 11041–8, A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood).

(1) Preparation of Recombinant Baculoviruses and Sf9 Cell Culture

A human SREBP 2 full length gene (Hua X, Yokoyama C, Wu J, Briggs M R, Brown M S, Goldstein J L, Wang X., Proc. Natl. Acad. Sci. U.S.A. 1993 December 15; 90(24): 11603–7., SREBP-2, a second basic-helix-loop-helix-leu-

cine zipper protein that stimulates transcription by binding to a sterol regulatory element) was integrated into a pBlue-Bac™ vector (Invitrogen, Carlsbad, Calif.). Sf9 cells (Invitrogen) were sub-cultured in Grace's supplemented media (GIBCO BRL) containing 10% fetal calf serum (Sigma), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 27° C. on a 10 cm diameter dish. Large scale culturing was performed in a 1L Spinner flask (Wheaton) with addition of 0.001% pluronic F-68 (GIBCO BRL). Recombinant baculoviruses were prepared according to the instructions (Bac-N-Blue™ Transfection Kit, Invitrogen). Recombinant baculoviruses were prepared by co-infecting Sf9 cells with Bac-N-Blue DNA (derived from ApMNPV) and 4 μg of pBlueBac-SREBP2.

(2) SDS-PAGE and Western Blot Analysis for Expression

0.83×10^6 cells/well of Sf9 cells were infected with the recombinant viruses prepared in (1) at MOI (multiplicity of infection) 5 in a 6-well dish. The cells and culture supernatant were collected periodically (24, 48, and 72 hours). After culturing for a certain period, Sf9 cells were scraped off with a cell scraper and centrifuged at 800 g for 10 minutes, thereby obtaining the cell fraction as a precipitate. The supernatant was stored as a culture supernatant fraction at −70° C. until sample preparation. The cell fraction was suspended in 100 μl/well of an isotonic phosphate buffer (a phosphate buffered saline containing 0.1% Triton X-100, aprotinin 0.5 μg/ml, leupeptin 0.5 μg/ml, pepstatin A 1 μg/ml), and then added with 100 μg/ml PMSF (phenylmethylsulfonyl fluoride). After vortex at 4° C. for 30 minutes and centrifugation at 1000 g for 10 minutes, 20 μl of 5×SDS sample buffer (0.24M Tris-HCl, pH6.8, 2.25% β-mercaptoethanol, 2.25% SDS, 50% glycerol, and 0.0015% bromophenol blue) was added to 80 μl of the supernatant, followed by heat treatment at 95° C. for 10 minutes. To 80 μl of the culture supernatant fraction was added 20 μl of a 5×SDS sample buffer, and the mixture was subjected to heat treatment.

These samples were subjected to gel electrophoresis on 8% SDS-PAGE and then transferred to a nitrocellulose membrane (Highbond ECL, Amersham) at 50V for 2 hours. After blocking with blockase for 30 minutes, the transfer membrane was allowed to react at room temperature for 1 hour with a 3000-fold diluted mouse ascite authentic sample of monoclonal antibody 1C6 (ATCC No CRL-2224) which recognizes the carboxyl end of SREBP 2. Then, the membrane was washed three times with TBS (20 mM Tris-buffered saline, pH 7.4), allowed to react with a peroxidase-bound anti-mouse IgG antibody (Sigma) for 1 hour, and washed with TBS. The membrane was then allowed to chemically emit with Supersignal west dura (Pierce), exposing to X-ray film.

(3) Sf9 Large Scale Suspension Culture and Sucrose Density Gradient Centrifugation of Budded Viruses

Sf9 cells were added at a concentration of 5×10^8 cells/500 ml in a Grace's supplemented medium containing 10% FCS, 0.001% PluronicF-68 (Gibco) in 1L spinner flask, and the cells were infected with SREBP 2 recombinant viruses at MOI 5 and cultured for 72 hours. The cells were removed by centrifugation at 800 g for 10 minutes, and the supernatant was ultra-centrifuged at 40,000 g for 20 minutes. The precipitate was suspended in 4 ml of a TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). 36 ml of a sequential sucrose gradient of 25% to 56% was prepared with TE buffer in tubes of a Beckman supercentrifuge SW28 rotor. The above virus suspension (1.2 ml) was placed onto the gradient, and centrifuged at 100,000 g for 90 minutes, and 1.5 ml fractions was collected from the top end of each centrifuge tube.

(4) Solubilization of SREBP 2

500 ml of the Sf9 culture cell suspension (1×10^9) was infected with SREBP2 recombinant viruses at MOI 5. 72 hours later, extracellular budded viruses (BV) were recovered by ultra-centrifugation at 40,000 g for 20 minutes. The pellet from the ultra-centrifugation was suspended in 4 ml of TBS, to which lyso-phosphatidylcholine (Sigma) was added to a concentration of 1%, followed by treatment at room temperature for 2 hours. The mixture was centrifuged at 30,000 rpm for 20 minutes with a Beckman rotor 90 Ti, so that a supernatant and a precipitate were separately recovered. The solubilized proteins are recovered in the supernatant.

(5) Affinity Chromatography

40 mg of IgG was purified from 4L of the culture supernatant of hybridomas (ATCC) secreting 1C6 with a Protein G column (Pharmacia), and was coupled to 8 ml of CNBr activated-sepharose (Pharmacia) to prepare a 1C6 affinity chromatography column.

The solubilized proteins recovered in the supernatant fraction were subjected to buffer exchange with buffer A (20 mM HEPES buffer containing 20 mM Octyl-glucose, pH7.4) using a PD10 column (Pharmacia), and then was applied to 2 ml of the above 1C6 affinity chromatography column, washed with buffer A containing 0.5M NaCl, and then eluted with buffer A containing 10M urea. The elution fraction was exchanged with buffer A using PD10 column again, and applied to a MonoS column (Pharmacia), followed by elution with a linear gradient of NaCl (0 to 0.5M) on Pharmacia SMART system.

(6) Expression of SREBP2

The amount of expression was confirmed by Western blot using a monoclonal antibody 1C6 (ATCC, USA) which recognizes the carboxyl end (FIG. 1). As a result, expression was confirmed in Sf9 cells 24 hours after reaction and in the culture supernatant 48 hours after reaction. To confirm the source of SREBP2 which has been recovered in the culture supernatant 48 hours after reaction, the culture supernatant was centrifuged. SREBP2 was recovered in the supernatant by centrifugation at 800 g for 30 minutes, and recovered in the pellet fraction by centrifugation at 40,000 g for 20 minutes (FIG. 2). These results suggest that SREBP2 present in the culture supernatant is not debris such as dead cells, but is derived from membrane or extracellular virus. Further, when the pellet fraction was fractionated by sucrose density gradient centrifugation, SREBP2 protein was recovered in the same fraction as that of the virus envelope protein gp64 which is confirmed by SDS-PAGE with Coomassie staining (FIG. 3). This result suggests that SREBP2 is not debris of cell membranes, but is expressed in the virus.

Moreover, when the fraction was treated with 1% lysolecithin, approximately 80% of SREBP2 was recovered in the soluble fraction. SREBP2 can be purified by a 1C6 affinity column in which the antibody 1C6 has been coupled to CNBr sepharose (FIG. 4).

(7) Immunization of Mice

1×10^9 cells/500 ml of Sf9 suspension cells were infected with SREBP2 recombinant viruses at MOI 5. 72 hours later, budded baculoviruses (BV) were suspended and recovered by ultra-centrifugation in 4 ml of phosphate buffered saline

(PBS). Mice were divided into three groups, each of which is of 2 mice, and immunized with virus solutions of 0.1 μl, 1 μl, and 10 μl, respectively. It was confirmed that anti-sera is produced upon two times of immunization (FIG. 5).

Example 2

Expression of S1P

S1P is a protease of one-transmembrane type which cleaves a precursor of sterol regulatory element binding protein SREBP (Sakai J, Rawson R B, Espenshade P J, Cheng D, Seegmiller A C, Goldstein J L, Brown M S., Mol Cell. 1998 October; 2(4): 505–14., Molecular identification of the sterol-regulated luminal protease that cleaves SREBPs and controls lipid composition of animal cells). S1P recognizes the RSVL sequence in the ER intraluminar loop of SREBP and cleaves it at the carboxyl end of a leucine residue. After synthesis in ER as a pro form (148 kd) consisting of 1052 amino acids, the signal peptide is cut off to produce S1P type A(120 kd). Subsequently, S1P type A is transported to Golgi body and is activated by auto-decomposition, resulting in S1P type B (106 kd) or type C (98 kd) (Espenshade P J, Cheng D, Goldstein J L, Brown M S., J Biol Chem. 1999 August 6; 274(32): 22795–804., Autocatalytic processing of site-1 protease removes propeptide and permits cleavage of sterol regulatory element-binding proteins). S1P has been also reported as a processing enzyme of brain-derived neurotrophic factor (BDNF) (Seidah N G, Mowla S J, Hamelin J, Mamarbachi A M, Benjannet S, Toure B B, Basak A, Munzer J S, Marcinkiewicz J, Zhong M, Barale J C, Lazure C, Murphy R A, Chretien M, Marcinkiewicz M., Proc. Natl. Acad. Sci. U.S.A. 1999 February 16; 96(4): 1321–6. Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization).

As in Example 1, a vector was constructed by integrating human S1P cDNA (KIAA0091, Kazusa DNA Research Institute) into pBlueBachis2™. Sf9 cells were co-infected with this vector and ApMNPV DNA (BAC-N-BLUE™, Invitrogen), thereby obtaining recombinant viruses. High Five™ cells (Invitrogen) were suspension- and sub-cultured in Express Five SFM (Gibco) supplemented with 16.5 mM L-glutamine.

High Five™ cells (Invitrogen) were infected with the recombinant viruses at MOI 5, and then extracellular budded viruses (BV) were prepared as described in Example 1. A partial amino acid sequence 589–604 of human S1P was synthesized, conjugated to Keyhole Limpet Hemocyanin, and used to immunize a rabbit, thereby obtaining anti-serum R03.

Expression of S1P in BV was examined using the thus prepared BV by immunoblotting with R03. As in Example 1, S1P was recovered in the culture supernatant centrifugation fraction (BV fraction) on and after 48 hours of culturing (FIG. 6).

Example 3

Measurement of Membrane Protease Activity

S1P is an enzyme which cleaves at almost the center of the 125 kd precursor protein of SREBP as described in Example 2. The cleavage site contains an RXXL sequence. Cleavage occurs at the carboxyl end of the leucine (L) residue. In SREBP2, the cleavage site of S1P which is RSVL is localized between two transmembrane sites at almost the center of the loop protruding into ER cavity. The cleavage is thought to occur in Golgi cavity when cholesterol content of cells decreases (Brown M S, Goldstein J L., Proc. Natl. Acad. Sci. USA, 1999 September; 96: 11041–11240., A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood).

If SREBP2 was expressed in the extracellular virus (BV) envelope in the correct orientation in Example 1, this would suggest that the cleavage site of SREBP2 was orientated towards the outside the virus. Similarly in Example 2, if S1P was expressed in BV in the correct orientation, this would suggest that the active site is located on the outside of the virus. To confirm if cleavage of SREBP2 by S1P occurred, both the virus solutions were mixed and incubated.

(Methods and Results)

Measurement of SREBP2 Cleavage Activity of S1P

Sf9 cells were infected with recombinant viruses of S1P or SREBP at MOI 5. 72 hours later, the BV fraction was recovered by centrifugation as in Examples 1 and 2, and then suspended in TBS buffer (10 mM Tris, 150 mM NaCl, pH7.5). BV suspended in TBS was subjected to a protein determination with a protein determination kit (Bio-Rad). Reaction solutions of SREBP2-BV as a substrate and S1P-BV as an enzyme were prepared to have respective final concentrations of 10 μg per reaction solution. In addition, calcium ions required for activation of S1P were added to the reaction solution at a concentration, as calcium acetate, which enables to measure activity. Calcium, EDTA (pH7.4) and EGTA (pH7.4) were added to BV solution, and then added with TBS buffer (pH7.4) to a reaction volume of 20 μl, followed by incubation at 37° C. for 12 hours. Following reaction, the solution was subjected to heat treatment with 5×SDS sample buffer at 95° C. for 10 minutes, subjected to 8% SDS-PAGE and Western blot using rabbit anti-serum R004 against the amino terminal of SREBP2.

Measurement of SREBP2 Cleavage Activity of S1P

BV expressing SREBP or S1P was mixed, incubated at 37° C. for 12 hours, and then allowed to react in the presence of 5 mM calcium. Western blot analysis with anti-serum R004 detected two major bands that were thought to be degradation products of SREBP (FIG. 7). Molecular weights of the bands which were thought to be degradation products were approximately 78 kDa and 64 kDa as calculated based on protein mobility (Rf value). The molecular weight of 64 kDa coincided with that of a degradation product (N terminus) resulting from cleavage of SREBP-2 by S1P as reported. Further, this degradation was completely inhibited by 10 mM EDTA. These results agreed with the S1P properties that have been reported (Toure, B. B., Munzer, J. S., Basak, A., Benjannet, S., Rochemont, J., Lazure, C., Chretien, M. and Seidah, N. G. (2000): Biosynthesis and Enzymatic Characterization of Human SKI-1/S1P and the processing of its inhibitory prosegment. J. Biol. Chem. 275, 2349–2358). However, a degradation product of 78 kDa has not been reported. Further purification and analysis are needed to know whether another cleavage site is present or this degradation is observed only in vitro.

Example 4

Expression of SCAP and Co-expression of SREBP2 and SCAP

SCAP (SREBP cleavage activating protein) is a membrane protein of eight-transmembrane type which has a

sterol sensor domain (SSD), and is present in ER membrane while forming a hetero-dimer with SREBP (Loisel T P, Ansanay H, St-Onge S, Gay B, Boulanger P, Strosberg A D, Marullo S, Bouvier M., Nat. Biotechnol. 1997 November; 15(12): 1300–4., Recovery of homogeneous and functional beta 2-adrenergic receptors from extracellular baculovirus particles). SSD senses a decrease in cholesterol level and guides SREBP towards the Golgi body where S1P is distributed, by an unknown mechanism (DeBose-Boyd R A, Brown M S, Li W P, Nohturfft A, Goldstein J L, Espenshade P J., Cell. 1999 December 23; 99(7)703–12., Transport-dependent proteolysis of SREBP: relocation of site-1 protease from Golgi to ER obviates the need for SREBP transport to Golgi). As described above, SCAP is known as an activator which activates a transport of SREBP or a cleavage enzyme depending on cholesterol.

Expression of SCAP

A vector was constructed by integrating a human SCAPcDNA (KIAA 0199, Kazusa DNA Research Institute) into pBlueBachis2™ (Invitrogen). Sf9 cells were co-infected with this vector and ApMNPV DNA (BAC-N-BLUE™, Invitrogen), thereby obtaining recombinant viruses. Sf9 cells were infected with the recombinant viruses at MOI5 as in Example 1. Immunoblotting with an antibody against His-tag (Qiagen) revealed that SCAP expressed in Sf9 cells aggregated but SCAP expressed in BV showed a correct electrophoretic mobility(FIG. 8).

Co-expression of SREBP2 and SCAP

SREBP2 and SCAP interact with each other at their carboxyl termini to form a hetero-dimer. This complex has been thought to be important in controlling cholesterol (Sakai J, Nohturfft A, Cheng D, Ho Y K, Brown M S, Goldstein J L., J. Biol. Chem. 1997 August 8; 272(32): 20213–21., Identification of complexes between the COOH-terminal domains of sterol regulatory element-binding proteins (SREBPs) and SREBP cleavage-activating protein).

Sf9 cells were co-infected at MOI5 with the recombinant virus of SREBP2 and that of SCAP prepared in Example 1. 72 hours later, BV was recovered from 200 ml of the culture supernatant and suspended in 4 ml of TBS. 1 ml of the suspension was added with lyso-phosphatidylcholine at a concentration of 1% (Sigma) and then treated at room temperature for 2 hours in the same manner as in the solubilization of SREBP2 in Example 1. Then, centrifugation was performed at 10,000 g for 10 minutes at 4° C. to separate and recover the supernatant and the precipitate. The supernatant was a soluble fraction and the precipitate was made into pellet after solubilization. 500 μl of the soluble fraction was dispensed into each of two 1.5 ml tubes, to which 30 μl of 1C6 affinity sepharose or 30 μl of Ni-NTA agarose (Qiagen) was added. The mixture was stirred at 4° C. for 16 hours. Each tube was centrifuged at 300 g for 3 minutes, resulting in the supernatant as an immunoprecipitation supernatant and the precipitate as an immunoprecipitation pellet. The immunoprecipitation supernatant (200 μl) was subjected to acetone precipitation (5 volumes of acetone was added, allowed to stand at −20° C. for 30 minutes, and then centrifuged at 3,000 g for 20 minutes). The product was suspended in 80 μl of a dissolution buffer (10 mM Tris-HCl, pH6.8, 100 mM NaCl, 1% SDS, 1 mM sodium EDTA, 1 mM sodium EGTA), to which 20 μl of 5×SDS sample buffer was added, followed by heat treatment at 95° C. for 5 minutes, thereby obtaining a sample for SDS-PAGE (Lanes 4 and 5 of FIG. 9).

The immunoprecipitation pellet was washed by suspending in 500 μl of TBS containing 0.5% lyso-phosphatidylcholine, and stirring at 4° C. for 16 hours. This was a first washing. Second and third washings were performed by adding the same amount of buffer and stirring for 1 hour. The supernatant was removed by centrifugation at 300 g for 3 minutes. After washing, the immunoprecipitation pellet was suspended in 100 μl of a 2×SDS sample buffer (60 mM Tris-HCl, pH6.8, 10% β-mercaptoethanol, 6% SDS, 10% glycerol, 0.008% bromophenolblue). Following heat treatment at 95° C. for 10 minutes, centrifugation was performed at 1000 g for 10 minutes, thereby recovering the supernatant as a sample for SDS-PAGE (lanes 6 and 7 of FIG. 9).

SDS-gel electrophoresis and immunoblot staining were performed in the same manner as in Example 1. Each sample for SDS-PAGE was subjected to gel electrophoresis on 7.5% SDS-PAGE, transferred to a nitrocellulose membrane at 70V for 2 hours, and then Western blot was performed. SREBP and SCAP were detected with the antibody 1C6 or the antibody against His-tag. Ni-NTA Agarose resulted in co-precipitation of SREBP and SCAP, suggesting that SREBP and SCAP formed complexes also in the baculovirus expression system (FIG. 9). In the 1C6 affinity Sepharose, only SREBP which formed no complex was precipitated, suggesting that 1C6 became incapable of recognizing an antigen site because of the formation of a complex of SREBP and SCAP.

INDUSTRIAL APPLICABILITY

Unlike membrane proteins which are recovered from Sf9 cell nuclei together with many degradation products, a highly stable endoplasmic reticulum membrane protein can be recovered in the viral envelope and recovered as a single band according to a method of the present invention, which expresses, in budded baculovirus, a protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, a protein involved in adhesion, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein. Although preparation of membrane proteins (particularly endoplasmic reticulum membrane proteins) is generally difficult because they are exposed to degradation enzymes such as lysozome when separating from a cell, membrane proteins expressed by the method of the present invention in extracellular viral envelopes can be easily prepared and possess good stability. The method of the present invention can be utilized for producing specific antibodies, purifying membrane proteins, measuring interaction between membrane proteins, and the like.

What is claimed is:

1. A method for recovering a budded baculovirus expressing an intracellular organelle membrane-bound protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, or a protein involved in formation of high dimensional structure of a protein comprising culturing a host infected with at least one recombinant baculovirus which contains a gene encoding said protein, expressing said protein in said infected host allowing baculovirus produced in said host to bud and be released from said host with said expressed protein being in the envelope of said budded baculovirus, and separating the budded baculovirus.

2. A method for preparing an intracellular organelle membrane-bound protein which comprises:

culturing a host infected with a recombinant baculovirus which contains a gene encoding protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, or a protein involved in formation of high dimensional structure of a protein;

recovering a budded baculovirus released from said host; and

recovering the protein expressed in said infected host allowing baculovirus produced in said host to bud and be released from said host with said expressed protein being in the envelope of said budded baculovirus.

3. The method of claim 1 wherein the protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, or a protein involved in formation of high dimensional structure of a protein is a membrane-bound protein of a cell organelle.

4. The method of claim 2 wherein the protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, or a protein involved in formation of high dimensional structure of a protein is a membrane-bound protein of a cell organelle.

5. The method of claim 1 wherein the protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, or a protein involved in formation of high dimensional structure of a protein is SREBP2, HMG-CoA reductase, S1P, or SREBP cleavage activating protein.

6. The method of claim 2 wherein the protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, or a protein involved in formation of high dimensional structure of a protein is SREBP2, HMG-CoA reductase, S1P, or SREBP cleavage activating protein.

7. The method of claim 1 wherein the host is an insect cell or an insect larva.

8. The method of claim 2 wherein the host is an insect cell or an insect larva.

9. The method of claim 1, wherein the protein is an Endoplasmic Reticulum-associated protein.

10. The method of claim 1, wherein the protein is an Golgi Apparatus-associated protein.

11. The method of claim 2, wherein the protein is an Endoplasmic Reticulum-associated protein.

12. The method of claim 2, wherein the protein is an Golgi Apparatus-associated protein.

13. The method of claim 1, wherein the protein is SREBP2, HMG-CoA reductase, S1P, or SREBP cleavage activating protein.

14. The method of claim 2, wherein the protein is SREBP2, HMG-CoA reductase, S1P, or SREBP cleavage activating protein.

15. A method for recovering a budded baculovirus expressing a non-receptor protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a membrane structural protein, or a protein involved in formation of high dimensional structure of a protein comprising culturing a host infected with at least one recombinant baculovirus which contains a gene encoding said protein, expressing said protein in said infected host allowing baculovirus produced in said host to bud and be released from said host with said expressed protein being in the envelope of said budded baculovirus, and separating the budded baculovirus.

16. The method of claim 15, wherein the protein is an Endoplasmic Reticulum-associated protein.

17. The method of claim 15, wherein the protein is an Golgi Apparatus-associated protein.

18. The method of claim 15, wherein the protein is SREBP2, HMG-CoA reductase, S1P, or SREBP cleavage activating protein.

19. A method for preparing a non-receptor protein which comprises:

culturing a host infected with a recombinant baculovirus which contains a gene encoding protein selected from a membrane-bound enzyme, a substrate of the membrane-bound enzyme, a membrane-bound enzyme activator, a membrane-bound transport protein, a channel protein, a protein involved in antigen presentation, or a protein involved in formation of high dimensional structure of a protein;

recovering a budded baculovirus released from said host; and

recovering the protein expressed in said infected host allowing baculovirus produced in said host to bud and be released from said host with said expressed protein being in the envelope of said budded baculovirus.

20. The method of claim 19, wherein the protein is an Endoplasmic Reticulum-associated protein.

21. The method of claim 19, wherein the protein is an Golgi Apparatus-associated protein.

22. The method of claim 19, wherein the protein is SREBP2, HMG-CoA reductase, S1P, or SREBP cleavage activating protein.

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